"A pilot study to look at the effect of IL-28B polymorphism on IL-28 expression and immunological recovery among HIV-1 infected individuals following Antiretroviral Therapy"

Dissertation submitted as part of fulfillment for the M.D. **(Branch-IV Microbiology)** Degree examination of the Tamil Nadu Dr. M.G.R. Medical University, to be held in **April 2015**

CERTIFICATE

This is to certify that the dissertation entitled **"A pilot study to look at the effect of IL-28 polymorphism on IL-28B expression and immunological recovery among HIV infected individuals following Antiretroviral Therapy"** is the bonafide work of **Dr. Srinidhi. B.V.** towards the MD**(Branch –IV Microbiology)** degree examination of the Tamil Nadu Dr. M.G.R Medical University to be conducted in **April 2015**.

Dr. Rajesh K,
Guide,
Professor,
Department of Clinical Virology,
Christian Medical College,
Vellore – 632004

Dr. V. Balaji,
Professor and Head
Department of Clinical Microbiology,
Christian Medical College,
Vellore – 632004

Dr. Balaji Veeraraghavan, MD, PhD.
Professor & Head of the Department
Dept. of Clinical Microbiology,
Christian Medical College & Hospital
Vellore - 632 004 South India

for **Principal,**
Christian Medical College,
Vellore- 632004

Principal
Christian Medical College
Vellore - 632 002, S.INDIA

DECLARATION

I hereby declare that this Dissertation titled **"A pilot study to look at the effect of IL-28 polymorphism on IL-28B expression and immunological recovery among HIV infected individuals following Antiretroviral Therapy"** is the bonafide work done by me under the guidance of **Dr. Rajesh Kannangai**, Professor, Department of Clinical Virology, Christian Medical College, Vellore towards M.D **(Branch –IV Microbiology)** Degree examination of the Tamil Nadu Dr. M.G.R Medical University, to be conducted in **April 2015**. This work has not been submitted to any other university in part or full.

Dr. Srinidhi. B.V.

PG Registrar,

Department of Clinical Microbiology,

Christian Medical College,

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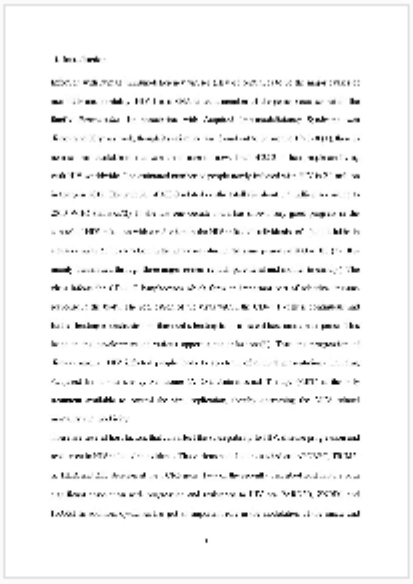

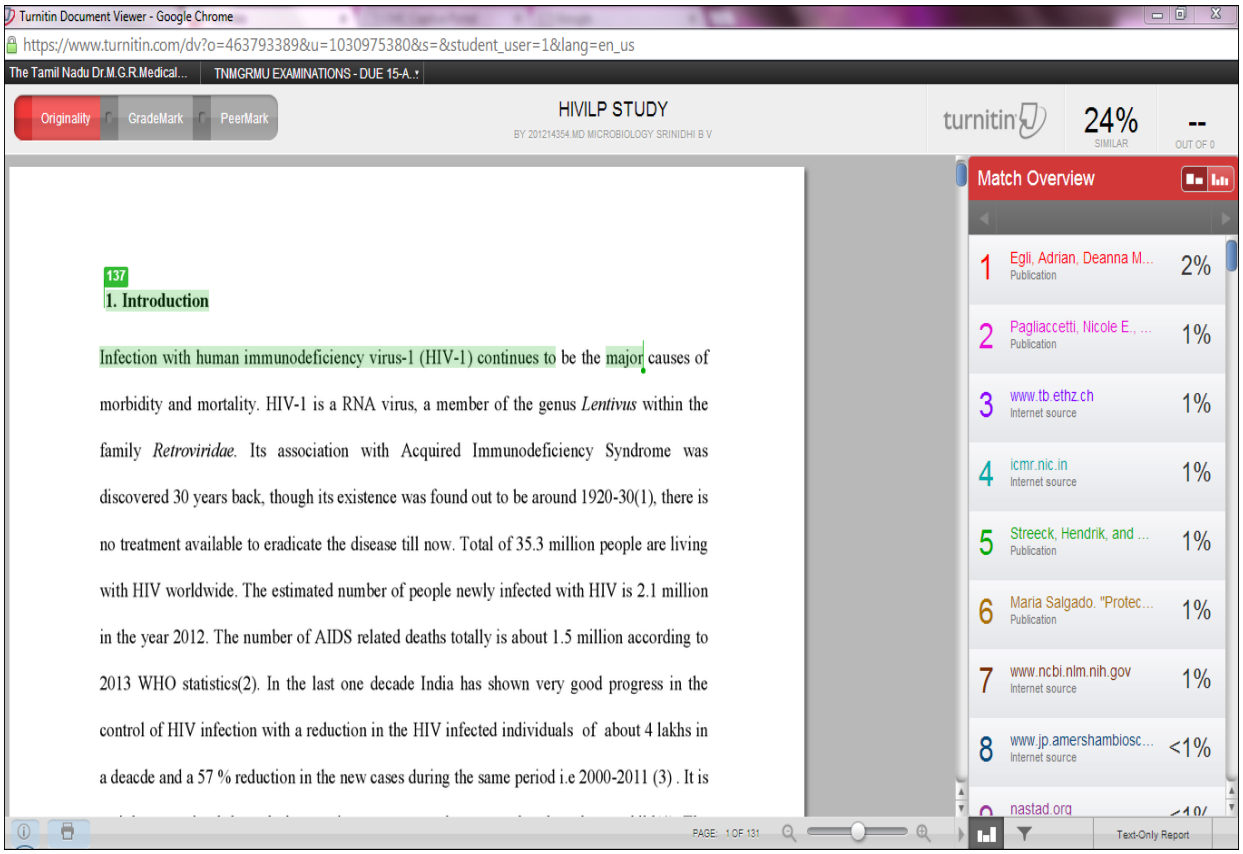
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1. Introduction
Infection with human immunodeficiency virus-1 (HIV-1) continues to be the major causes of morbidity and mortality. HIV-1 is a RNA virus, a member of the genus Lentivus within the family Retroviridae. Its association with Acquired Immunodeficiency Syndrome was discovered 30 years back, though its existence was found out to be around 1920-30(1), there is no treatment available to eradicate the disease till now. Total of 35.3 million people are living with HIV worldwide. The estimated number of people newly infected with HIV is 2.1 million in the year 2012. The number of AIDS related deaths totally is about 1.5 million according to 2013 WHO statistics(2). In the last one decade India has shown very good progress in the control of HIV infection with a reduction in the HIV infected individuals of about 4 lakhs in a deacde and a 57 % reduction in the new cases during the same period i.e 2000-2011 (3) . It is
PAGE: 1 OF 131
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I would like to express my gratitude to the following people for their contributions during this study:

Dr. Rajesh Kannangai, for his constant supervision, guidance and support throughout my study.

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My parents, my lady charm Chaitra and my in-laws for their emotional support and encouragements during this stressful period of completing the Dissertation.

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Last but not least, I would like to thank the Department of Clinical Virology and the Institutional Review Board, Christian Medical College, Vellore for funding my Dissertation.

I would like to dedicate this study to my parents, teachers, patients who taught me and and healthy individual and to everyone involved in this study.

I will be missing the cute bands of RFLP and my pal BD FACS machine!

Dissertation is not a highway ride, drive accordingly to overcome the speed breakers to reach your real destiny!

Above all, I thank god for his support in completing this study.

Table of Contents

List of Tables:

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Additional Vice Principal (Research)

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Dr. Srinidhi B V
PG Registrar
Department of Clinical Microbiology
ChristianMedicalCollege
Vellore 632 002

Sub: **FLUID Research grant project NEW PROPOSAL:**
A pilot study to look at the effect of IL-28 polymorphism on IL-28 expression and immunological recovery among HIV infected individuals following ART.
Dr. Srinidhi B V, PG Registrar, Department of Clinical Microbiology,
Dr. Rajesh Kannangai , Clinical Virology, Dr. O C Abraham, Medicine I,
Dr. Susanne A Pulimood , Dermatology, Dr. Priscilla Rupali , Medicine I,
Dr. John Fletcher G, Mr. Jaiprasath Sachithanandham , Clinical Virology.

Ref: IRB Min. No. 8237 dated 19.03.2013

Dear Dr. Srinidhi B V,

The Institutional Review Board (Blue, Research and Ethics Committee) of the ChristianMedicalCollege, Vellore, reviewed and discussed your project entitled "A pilot study to look at the effect of IL-28 polymorphism on IL-28 expression and immunological recovery among HIV infected individuals following ART." on March 19, 2013.

The Committees reviewed the following documents:

1. Format of IRB application form
2. Information Sheet and informed Consent Form (English, Hindi, Tamil and Telugu)
3. Proforma
4. Cvs of Drs. Srinidhi B V, Rajesh Kannangai, O C Abraham, Susanne A Pulimood, Priscilla Rupali, John Fletcher G, Mr. Jaiprasath Sachithanandham.
5. A CD containing documents 1 – 3

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Dr. John Fletcher G, Mr. Jaiprasath Sachithanandham , Clinical Virology.

Ref: IRB Min. No. 8237 dated 19.03.2013

Dear Dr. Srinidhi B V,

I enclose the following documents:-

1. Institutional Review Board approval
2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
Secretary (Ethics Committee)
Institutional Review Board

CC: Dr. Rajesh Kannangai, Department of Virology

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The following Institutional Review Board (Research & Ethics Committee) members were present at the meeting held on March 19, 2013 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Name	Qualification	Designation	Other Affiliations
Dr. Susanne Abraham	MBBS, MD	Professor, Dermatology, Venerlogy & Leprosy, CMC.	Internal, Clinician
Dr. Benjamin Perakath	MBBS, MS, FRCS	Professor, Surgery (Colorectal), CMC.	Internal, Clinician
Dr. Ranjith K Moorthy	MBBS MCh	Professor, Neurological Sciences, CMC	Internal, Clinician
Dr. P. Prasanna Samuel	B.Sc, M.Sc, PhD	Professor Dept. of Biostatistics, CMC	Internal, Statistician
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Dept. of Pulmonary Medicine, CMC.	Internal, Clinician
Dr. Anup Ramachandran	PhD	The Wellcome Trust Research Laboratory Gastrointestinal Sciences	Internal
Dr. Chandrasingh	MS, MCH, DMB	Urology, CMC	Internal, Clinician
Dr. Paul Ravindran	PhD, Dip RP, FCCPM	Professor, Radiotherapy, CMC	Internal
Dr. Anil Kuruvilla	MBBS, MD, DCH	Professor, Neonatology, CMC.	Clinician
Dr. Ellen Ebenezer Benjamin	M.Sc	Maternity Nursing, CMC	Internal, Nurse

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Mr. Joseph Devaraj	BSc, BD	Chaplain, CMC	Internal, Social Scientist
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Dr. Jayaprakash Muliyil	BSC, MBBS, MD, MPH, DrPH(Epid), DMHC	Retired Professor, Vellore	External, Scientist
Dr. Nihal Thomas	MD MNAMS DNB(Endo) FRACP(Endo) FRCP(Edin)	Secretary IRB (EC)& Dy. Chairperson (IRB), Professor of Endocrinology & Addl. Vice Principal (Research), CMC.	Internal, Clinician

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MD,MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
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We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any serious adverse events occurring in the course of the project, any changes in the protocol and the patient information/informed consent. And on completion of the study you are expected to submit a copy of the final report.

A sum of Rs 60,000/- (Rupees Sixty Thousand only) will be granted for 18 months.

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
Secretary (Ethics Committee)
Institutional Review Board

CC: Dr. Rajesh Kannangai, Department of Virology

TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

List of Abbreviations :

HIV – Human Immunodeficiency Virus

ART - Anti-Retroviral Therapy

HAART – Highly Active Anti-Retroviral Therapy

PCR – Polymerase Chain Reaction

IL – Interleukins

PBMC – Peripheral Blood Mononuclear Cells

HCV – Hepatitis c virus

SNP – Single nucleotide polymorphism

IFN-λ – Interferon lambda

STAT 1 and STAT 2 – Signal Transducer and Activator of Transcription

IRF9 – Interferon Response Factor 9

ISGF3 – Interferon Stimulated Gene Factor 3

ISG – Interferon Stimulated Genes

PEG-IFN/RBV – Pegylated Interferon and Ribavarin

IRIS – Immune Reconstitution Inflammatory Syndrome

CpG– Cytosine-phosphate-Gaunine

Abstract

Title of the abstract: "A pilot study to look at the effect of IL-28B polymorphism on IL-28 expression and immunological recovery among HIV-1 infected individuals following Antiretroviral Therapy"

Department: Department of Clinical Microbiology

Name of the candidate: Dr. Srinidhi B V

Degree and subject: M.D Microbiology

Name of the guide: Dr. Rajesh K.

Objectives:

To look at the frequency of IL-28B polymorphisms in south Indian HIV infected individuals and its effect on IL-28 plasma level and immunological recovery following ART.

Methods:

A total of 49 HIV infected individuals and 30 healthy controls were recruited. Whole blood samples were collected before and after 6-9 months of ART from patients. Absolute CD4+/CD8+ T cell counts, CD3+cell counts and CD4/CD8 ratio were estimated using flow cytometry (FACS Count). IL-28B polymorphism at rs12979860 and rs8099917 were detected by PCR-RFLP and IL-28B plasma level estimation was done by ELISA. Association between polymorphism, cell counts and IL-28 plasma level were analyzed.

Results:

There was significant association of CC genotype at rs12979860 (p=0.03) and CC/TT haplotype (p=0.03) with higher CD4+T-cell count among treatment naïve HIV infected individuals. There was a significant (p=0.03) association of CC/TT haplotype with increase in CD4/CD3% following ART. There was no correlation (p=>0.05) between IL-28B plasma level with IL-28B polymorphism, CD4+ T cell and CD4/CD8 ratio. There was no significant difference in the frequencyof polymorphism and IL-28B plasma level between HIV infected individuals and healthy controls. The CT/GT haplotype had a significant higher IL-28B plasma level compared to wild type CC/TT before the initiation of ART and significantly higher decrease observed in CT/GT haplotype compared to CC/TT wild type were significant

Conclusion:

In conclusion our preliminary data from this pilot study showed significantly higher CD4+ T-cells among HIV infected individuals with wild haplotype (CC/TT) prior to ART and significantly high CD4+ T cells and CD4/CD3% following ART. This study showed no association of IL-28B polymorphism with IL-28B plasma level and CD4+T cell count or CD4/CD8 ratio. Since IFNλ is a powerful immune modulator functional studies are warranted to understand the IFNλ mediated immuno-pathogeneis in HIV infection.

AIMS AND OBJECTIVES

1) HYPOTHESIS

The IL-28 gene polymorphism(s) will enhance the expression of the interleukin-28 and the recovery of CD4+ T- cell following ART among HIV-1 infected individuals

2) AIM

To study the frequency and distribution of IL28B polymorphisms and its influence of IL28B plasma level and immunological recovery in HIV infected individuals following 6-9 months of Antiretroviral Therapy.

3) OBJECTIVES

1) To look at the frequency of IL-28B polymorphisms in HIV infected individuals and healthy controls.
2) To determine the IL-28B plasma level in the treatment naive HIV infected individual and healthy controls.
3) To determine the IL-28B plasma level in HIV infected individuals following ART.
4) To look at any association of IL-28B plasma level in HIV infected individuals including those presenting with Immune Reconstitution Inflammatory Syndrome (IRIS) following ART.
5) To analyze the association of IL-28B polymorphism with plasma level of IL-28B and immunological recovery (% increase in CD4+ T cells) following ART among HIV-1 infected individuals.

1. Introduction

Infection with human immunodeficiency virus-1 (HIV-1) continues to be the major causes of morbidity and mortality. HIV-1 is a RNA virus, a member of the genus *Lentivus* within the family *Retroviridae.* Its association with Acquired Immunodeficiency Syndrome was discovered 30 years back, though its existence was found out to be around 1920-30(1), there is no treatment available to eradicate the disease till now. Total of 35.3 million people are living with HIV worldwide. The estimated number of people newly infected with HIV is 2.1 million in the year 2012. The number of AIDS related deaths totally is about 1.5 million according to 2013 WHO statistics(2). In the last one decade India has shown very good progress in the control of HIV infection with a reduction in the HIV infected individuals of about 4 lakhs in a deacde and a 57 % reduction in the new cases during the same period i.e 2000-2011 (3) . It is mainly transmitted through three major routes; sexual, parenteral and mother to child(4). The virus infects the CD4+ T lymphocytes which form an important part of adaptive immune response in the body. The replication of the virus within the CD4+ T cells is continuous and further leading to destruction of these cells leading to insufficient host immune response. This leads to the development of various opportunistic infections(5). Thus the progression of disease among HIV infected people leads to spectrum of clinical presentations including Acquired Immunodeficiency Syndrome (AIDS). Antiretroviral Therapy (ART) is the only treatment available to control the viral replication, thereby decreasing the AIDS related mortality and morbidity.

There are several host factors that can affect the susceptibility to HIV, disease progression and resistance in HIV infected individuals. The various host factors studied are APOBEC, TRIM5-α, HLA and Δ32 deletion at the CCR5 gene. Few of the recently described host factors with significant association with progression and resistance to HIV are PARD3B, ZNRD1 and

PROX1.In addition; cytokines has got an important role in the modulation of the innate and adaptive immunity.. Various cytokines have also been found to affect the disease progression like IL-10, IL-21, TNFα, MIP1-α and RANTES.

IFN-λ is a newly discovered group of antiviral cytokine. IFN-λ subfamily is comprised of IFN-λ1, IFN-λ2, IFN-λ3 which are also called interleukin-29 and interleukin-28A, interleukin-28B respectively(6). It is documented to have antiviral activity in various viral infections(7).The antiviral activity on HIV is documented in few studies *in vitro*, which have reported that IFN-λ could inhibit the HIV replication.

Four landmark studies published in 2009 described a clinical association between the response to treatment with pegylated interferon and ribavirin(PEG-IFN-α) among hepatitis C virus (HCV) infection with IL28B polymorphism(8)(9). Both spontaneous HCV clearance and a sustained viral response following PEG-IFN-α plus ribavirin therapy correlated with wild type genotypes of CC at rs12979860 and TT at rs8099917(9)(10). A single nucleotide polymorphisms (SNPs) reported in the *IL28B* gene locus, that encodes the IFN-λ3 protein can modify this. The Role of IL-28B polymorphism in HIV infected individuals is not clear. Only few studies have documented its association in HIV infected individuals.

Studies on the association of IL-28B polymorphism in HIV patients are contradicting and limited. Serra *et al* ,(2008), showed that the CD4, CXCR4, and CCR5 protein expression increased when the PBMC pretreated with IFNλ-2 and is associated with enhanced binding and replication of HIV-1. Hou *et al* ,(2009), IFN-λ enhances the APOBEC3G and APOBEC3F expression at both the mRNA and protein levels in macrophage. Rallon et al ,2011 and Sajadi *et al* (2011), showed that the association of the IL-28B SNP with HIV progression of disease or protection agaist HIV (11)(12).

Based on the anti-HCV properties of IFN-λ and the association of IL-28B polymorphism with better treatment outcome in HCV infection, in this study we looked at the association of IL-

28B polymorphism and immune recovery among HIV-1 infected individuals following 6-9 months of antiretroviral therapy (ART). It is also important know the effect of these polymorphism and the IL-28B cytokine level both in the expression and plasma level as this cytokine s found to have antiviral effect. In this reported study an effort was also made in this direction.Based on the anti-HCV properties of IFN-λ and the association of IL-28B polymorphism with better treatment outcome in HCV infection, in this study we looked at its association in HIV infected individuals following 6-9 months of ART.

2. REVIEW OF LITERATURE

2.1. History of HIV

The origin of the AIDS pandemic began in June 1981 when the Centers for Disease Control and Prevention (CDC) in the USA published a report in the Morbidity and Mortality Weekly Report on five cases of rare *Pneumocystis carinii* pneumonia in previously healthy young homosexual men in Los Angeles (Centers for Disease Control, 1981b). More case reports by Gottlieb *et al* (1981) and Masur et al (1981) revealed that this disease being associated with a depletion of T-helper cells were described(13)(14). In that same year, reports of a rare and aggressive form of Kaposi's sarcoma in young homosexual men in both New York and California 15 appeared(15), which were also associated with a loss of T-helper cells (Stahl *et al* ., 1982)(16). This condition was later officially named Acquired Immunodeficiency Syndrome, or AIDS, by the end of 1982.

L. Montagnier and R. Gallo (Gallo and Montagnier, 2003) described their search for the causative agent of AIDS during the early years of the epidemic. As the cases of AIDS were observed to transmit through blood and sexual activity, from mother to child, as well as through filtered blood products containing clotting factors for haemophilia, a virus was thought to be the transmissible agent. The observations that the depletion of CD4+ T-helper cells was the biological marker in AIDS patients and AIDS-like wasting syndrome that was caused by lymphotropic retroviruses in animal models together led to a search for a retrovirus or a variant of the human leukemia virus (HTLV) as the causative agent of AIDS.

The association between AIDS and HIV was made by Barré -Sinoussi and Luc Montagnier at Pasteur Institute in May 1983.They described the isolation of a novel retrovirus from a lymph node of a homosexual patient with multiple lymphadenopathies(17). T lymphocytes from a healthy adult donor and from umbilical cord blood of newborn were used to propagate the

retrovirus. Viral core proteins were found to be not immunologically related to P24 and p19 proteins of subgroup I of HTLV. Reverse transcriptase activity was detected in the culture supernatant after 15 days of culturing the lymph node lymphocytes, indicating the presence of a retrovirus that was then observed under electron microscopy.

Antisera to HTLV-1 did not react with cells infected with the novel virus, indicating that the new virus was distinct from HTLV. A protein of similar size to the p24 core protein of HTLV-1 was found, but was not recognized by antibodies to the HTLV-1 p24 protein. The viral core proteins were not immunologically related to the p24 and p19 proteins of subgroup I of HTLV(17).

Based on these observations, Barré-Sinoussi *et al* ,1983. concluded that the 16 novel virus belonged to a general family of T lymphotropic retroviruses, which was related to but distinct from HTLV. The virus was subsequently called lymphadenopathy virus (LAV).

Vilmer et al ., 1984, reported the isolation of LAV from two siblings with Hemophilia B. In May 1984, Robert Gallo and his coworkers reported the isolation of a novel retrovirus from individuals with AIDS and subsequent studies showed the evidence of this retrovirus being the etiological agent for AIDS(18). Serological analysis of antigens of HTLV-III, detected specifically by antibodies in serum from AIDS or pre-AIDS patients by Western blot technique, revealed that they are similar in size to those found in other HTLV subgroups. Hence concluded that HTLV-III is a member of HTLV family(19). The virus was called human T-lymphotropic virus type III, or HTLV-III.

In August 1984, Jay Levy (Levy *et al*, 1984) reported an isolation of retroviruses from patients with AIDS and called it as AIDS-associated retrovirus, or ARV which had a type-D morphology, Mg^{2+} dependent reverse transcriptase and cytopathic effect on lymphocytes. They cross reacted with antiserum to LAV isolated from AIDS patients in France(20).

In 1985, molecular cloning and sequencing techniques showed that LAV, HTLV-3 and ARV are same species(21). The International committee on the taxonomy of the virus recommended that the terms LAV, HTLV-III and ARV should not be used and named it as Human Immunodeficiency Virus (HIV)(22).

2.2. GLOBAL BURDEN

Total of 35.3 million (33.1-37.2 million) people are living with HIV worldwide, of which adults are about 31.8million, women about 16.0 million and children(<15 years) about 3.2 million. The estimated number of people newly infected with HIV is 2.1 million (1.9-2.4 million) of which adults are about 1.9 million and children (<15 years) 240,000.The number of AIDS related deaths totally is about 1.5 million(1.4- 1.7million) with adults of about 1.3 million and children (<15 years) of about 190,000 according to 2013 WHO statistics. The worldwide prevalence of HIV among adults is as shown in the figure 1 below(2).

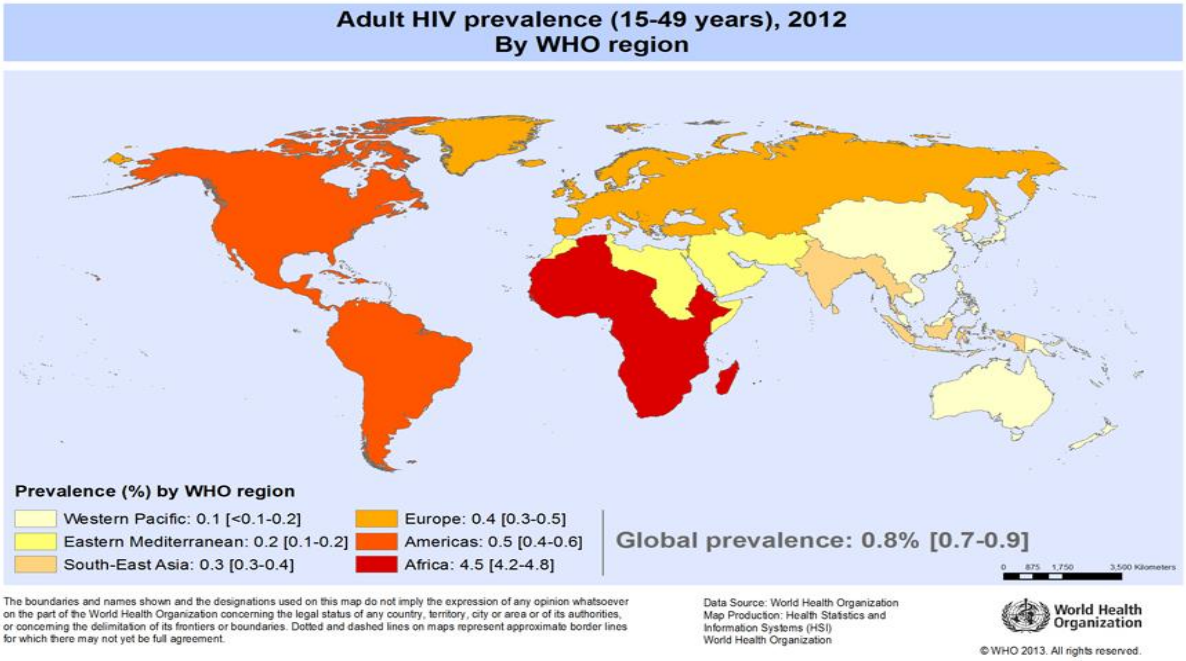


Figure: Global prevalence of HIV in different WHO regions (Source: WHO 2013).

2.3. INDIAN SCENARIO

The NACO annual report 2012 on Indian scenario revealed that there is an overall reduction of 57% in the newly infected adults, i.e from 2.74 lakhs in the year 2000 to 1.16 lakhs in the year 2011 in India. The adult HIV prevalence has decreased from 0.41% (2001) to 0.27% (2011). The number of people living with HIV is estimated to be decreased from 24.1 lakh in 2000 to 20.9 lakhs in 2011 according to NACO Annual report 2012-13(4). Eighty three percent of these infections occur in adults in the age group of 15-49 years.

A total of 6.04 lakh individuals living with HIV are receiving ART as of 2012-2013 National AIDS Control Organization (NACO) statistics. The high risk group of population includes female commercial sex workers, intravenous injection drug users, men having sex with men and transgenders. The prevalence is twenty times higher in risk groups. Heterosexual contributes to 87.4% of the infections and homosexual route contributes to 1.3%(4) . The infection is transmitted from the high risk group to the general population through the bridge population which includes the truck drivers and the migrant population (clients of sex workers). Married women are at risk of acquiring infection from their husbands who mostly get infected because of their promiscuous behavior(23). Studies have shown that serodiscordant couples are at risk of contracting the infection influenced by various behavioral patterns and socio-economic factors. Approximately 5.4% of the infections are due to transfer from mother to child and 1% due to parenteral transmission through blood and blood products (2). Needle sharing for intravenous drug abuse is the predominant mode in North Eastern states of India accounting to 1.6% of infections. (3).

The distribution across the states within the country is heterogeneous as shown in figure 2 below and the highest prevalence (1.4%) is noted in the north eastern states.

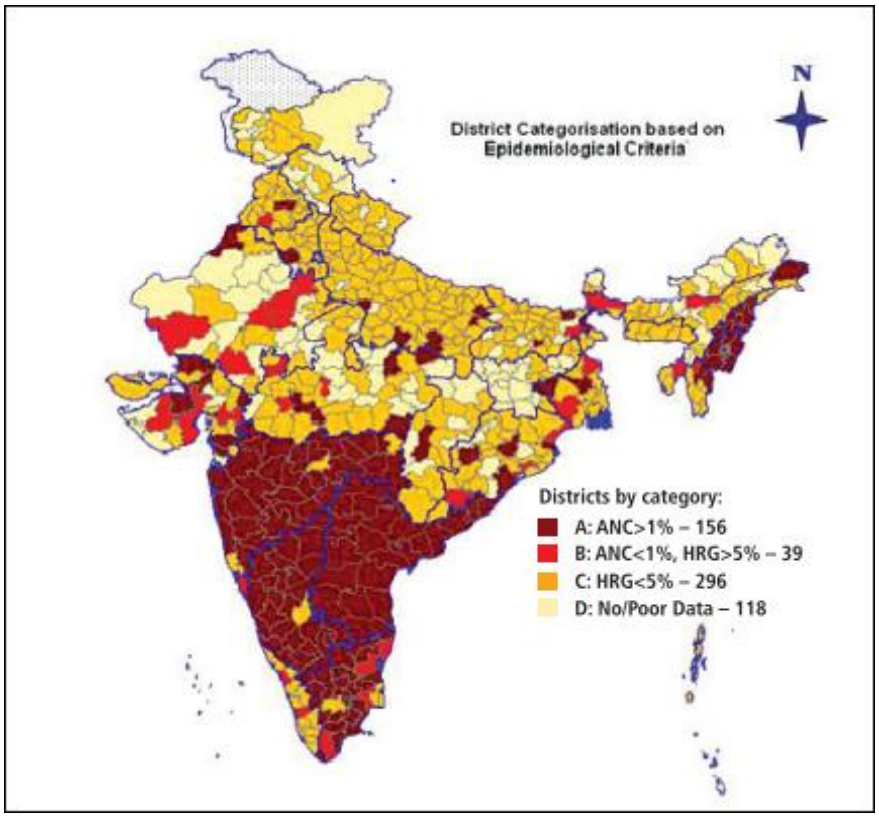


Figure 2: District-wise adult HIV prevalence in India in different states (Source: NACO Fact sheet 2013-14)

Tamil Nadu is one among the high risk states with the prevalence of 0.33%, it being higher than the national prevalence (24). The National AIDS Control Programme (NACP I-III) was introduced in India in the year 1992 for implementation of policies to combat the problem of HIV. NACP IV over 2012-17 aims to accelerate the process of reversal, further strengthening the epidemic response in India through a cautious and well-defined integration process. Main objective of NACP IV is to reduce new infections and provide comprehensive care and support to all PLHIV and treatment services to all those who require it. The introduction of targeted interventions and ART has changed the trend of HIV in India. The prevalence has reduced from 0.39% in 2004 to 0.27% in 2011. There are 380 ART centres and around 10000 Integrated Counseling and Testing Centres (ICTC) in India. These government funded centres provide pre and post test counseling, promote safe sexual practices and offer Highly Active Antiretroviral Therapy (HAART). There was a 29% reduction in the deaths due to AIDS in the five year period from 2007-2011(4). Also the rate of new infections among the

high risk group has stabilized as a result of awareness and education. Newer policies and strengthening of the existing programmes has made it possible to cause these changes.

2.4. DIVERSITY AND ORIGIN OF HIV

2.4.1. Source of HIV infection

The source of HIV-1 and HIV-2 infection to humans was postulated to be by several cross species transmissions of simian immunodeficiency viruses(SIVs) from chimpanzees(SIVcpz) and sooty mangabeys (SIVsm) respectively (25).It is also shown that SIVcpz can cause AIDS-like immunopathology in chimpanzees as SIVs were considered as non-pathogenic in their hosts (26).Later it was confirmed that HIV-1 was derived from SIVcpz from the Pan troglodytes troglodytes subspecies of chimpanzees (27). Another study showed that HIV-1 of group M (major or pandemic group) and N (non-major, non-outlier) arose from two distinct SIVcpz strains, circulating in two geographically separated chimpanzee populations present in west central Africa and HIV-1 of group O (outlier) as the result of a cross-species transmission of SIVgor in gorillas(28).

The earliest known HIV-1 seropositive infection was detected in 1959 from a plasma sample from an adult male Kinshasa from Democratic Republic of Congo(29). Phylogenetic analyses of HIV-1 and HIV-2 sequences have revealed that the origin of HIV-1 infection leading to the pandemic at present to around 1920-1930 in Central West Africa including the 1959 sample of HIV-1 and the origin of HIV-2 is estimated to 1940 in West Africa(1)(30).

Phylogenetically, HIV-1 can be classified into three divergent lineages arising from a separate transmission events from chimpanzees(31). Lineages are group M(major), N(non-major, non-outlier) and O(outlier). In 2009, a new HIV was identified in a Cameroonian woman which was closely related to gorilla Simian Immunodeficiency Virus(SIVgor) distinct

from HIV-1 groups M, N and O and designated it as HIV-1 group P(32).Group M is seen worldwide and can be classified into genetic subtypes. Groups N and O are limited to few individuals in Central Africa(31).The M group is the most prevalent in the world among the three groups. It has nine subtypes (A-D, F-H, J, K), all the subtypes have originated from Central Africa. The amino acid distances in the env gene between the subtypes in the major group is about 25-35% and in the gag gene it is about 15%.Within subtypes A and F there are sub clusters namely A1, A2 and F1, F2 respectively. Subtypes B, C and G have genetically localized sub-clusters which share common ancestry, Subtype B from Thailand, subtype C from India, Ethiopia and Brazil and subtype G from Spain and Portugal(33).

Recombinant forms are seen in geographic areas where more than one type is circulating are reported. If two different subtypes of HIV-1 infect a single one individuals , following replication a mosaic genome can be resulted comprising regions from the two subtypes. This is due to the "template switching" ability of the reverse transcriptase enzyme.

There are two types of recombinant forms–

1)Circulating Recombinant Forms (CRF) and the Unique Recombinant Forms (URF)(34). CRFs are the recombinants identified in at least three epidemiologically unlinked individuals characterized by full-length genome sequencing. There are currently 66 recognized CRFs and one for HIV-2(35). Majority of the CRFs reported are from Africa. The formerly designated subtypes E and I are now reclassified as CRFs.

2) Unique Recombinant Forms (URF) have not shown any evidence of epidemic spread and are thought to arise due to secondary recombination of a CRF. These HIV-1 strains with unique mosaic structures have been reported in epidemiologically linked persons. Currently there are about 30 of them

HIV is the most variable of human pathogens, and it exists as a swarm of highly related but non-identical viral genomes known as “quasispecies”.

The sources of this variation are due to these three factors:

1) The rapid replication rate of the virus, which is estimated to produce 10^{10} virions per day in an infected individual(36)

2) The high error rate of reverse transcriptase (RT) due to lack of proof reading.

3) High recombination rate due to the alternate copying from the two RNA molecules found in each virion(37).

There are two types of HIV spreading in the human population. HIV type-1 (HIV-1) is the cause of pandemic.HIV type-2(HIV-2) was discovered in 1985(38).HIV-2 is more commonly seen in West African countries like Senegal, Guinea, Guinea-Bissau and Senegal, prevalence being 1-10%.It is also found in countries with past socio-economical links with Portugal such as France, India, Angola, Mozambique and Brazil(39). In India a dual epidemic of HIV-1 and HIV-2 is ongoing with HIV-1 being the predominant one.

2.4.2. HIV-1 subtype C

Several genotype study carried out across India had shown the high prevalence of subtype C varying form 65% to 97.8 %(40).

High prevalence of subtype C is seen in India. It shares at present 50% of the global infections(41)(42). Subtype C demonstrates several interesting genotypic and phenotypic properties. Studies have shown that HIV-1 subtype C long terminal repeat (LTR) has a third NF-κB site whereas most non-C strains including subtype B viral strains have merely two NF-κB sites. Recent study in India, reported the presence of an additional 4th NF-κB in LTR, and four NF-κB strains are expanding and replacing the three NF-κB sites containing subtype C viruses. Individuals who are infected with viruses harboring four NF-κB sites had high

viremia compared to those individuals with virus containing only three NF-κB sites, although there was no significant difference in their CD4+ T-cell counts exists (43), which gave the conceptual premise that additional NF-κB in the HIV-1 subtype C LTR might enhance the viral replication competence by enhancing the infectivity.

Another important difference is in neuropathogenetic ability. HIV-associated dementia (HAD) is common among untreated HIV-1 subtype B-infected individuals, but less common in subtype C infections. Apart from this, several subtype C specific genetic signature residues were reported clinically important reverse transcriptase (RT) and protease (PR) region of Pol(44). Significant co-receptor related disparities also have been observed in HIV-1 subtype C. In the later stage of disease nearly 50% of the subtype B strains are X4-tropic, while in subtype C, majority the strains use exclusively the CCR5 co-receptor (45).

Significant disparity also has been observed in the clinical course of HIV-1 infection and development of drug resistance with subtype C viruses. Studies have shown that a substantial proportion of HIV-infected adults maintained a high viral set point after acute subtype C infection and might be responsible for rapid spread of this viruses (40). However, a recent study from East and Southern African countries showed that subtype C and non-C (A,D & other) subtypes do not differ significantly in terms of their viral load and rapid spread of subtype C (46). Thus the spread of HIV-1 subtype C strains is still inconclusive. However, there are likely that multiple factors including viral and host immuno-genetics as well as clinical and geographical disease management strategies can play a crucial role in the rapid spread and prevalence of HIV-1 subtype C strains globally(40).

2.5. HIV-1 Transmission

HIV-1 can be transmitted along three major routes. The first route is through direct exposure to HIV-1 positive blood or blood products, either in donated blood or contaminated needles, and represents about 5-10% of HIV-1 infections worldwide.

The second route is via mother-to-child transfer, and represents about 15% of new infections in (UNAIDS/WHO, 2013). The other route is through sexual contact primarily at the genital and rectal mucosa, and represents the vast majority of all new infections. NACO estimation showed 87.4% through heterosexual route and 1.3% through homosexual route(3). The probability of infection through sexual contact can vary greatly, and is dependent on the viral dose and also on whether the virus is transmitted directly into the blood via breaks in the epithelium or across intact mucous membranes.

Circumcision confers a reduced risk of HIV-1 infection for men, highlighting the important role of the foreskin in HIV-1 transmission (Gray et al ., 2007).Various factors can lead to an increased risk of HIV-1 transmission, such as physical abrasion, abnormalities in the vaginal flora or genital ulcers caused by sexually transmitted diseases(47).Although the events on how HIV-1 can lead to an established infection when it reaches the epithelium is not clearly understood, studies on explants models have shown that HIV-1 can directly infect Langerhans cells, subepithelial DCs, macrophages and CD4+ T cells(48).

The probability of HIV transmission depends on the amount of the infectious virus particles present in the body fluid, mainly blood and genital fluid in the index patient and the extent of exposure of that body fluid. Also, the susceptibility of the exposed individual is also clearly important. Generally, transmission events occurs among individuals with a blood viral load >3.5 Log10 copies/ mL. The individuals who are at risk of acquiring infection through this route include injection drug users (sharing of needles and syringes), those receiving blood

and blood products, transplanted organs. Injection drug users account for 1.6% of the infections in our country(3).

2.6. HIV-1 Structure

The average diameter of a mature HIV-1 virion is about 145 nm (49) and is enveloped with host cell-derived lipid bilayer embedded with virus-encoded Env consisting of the surface glycoprotein gp120 non-covalently linked to the transmembrane glycoprotein gp41(49).The diagrammatic representation of structure of HIV is shown in figure 3.

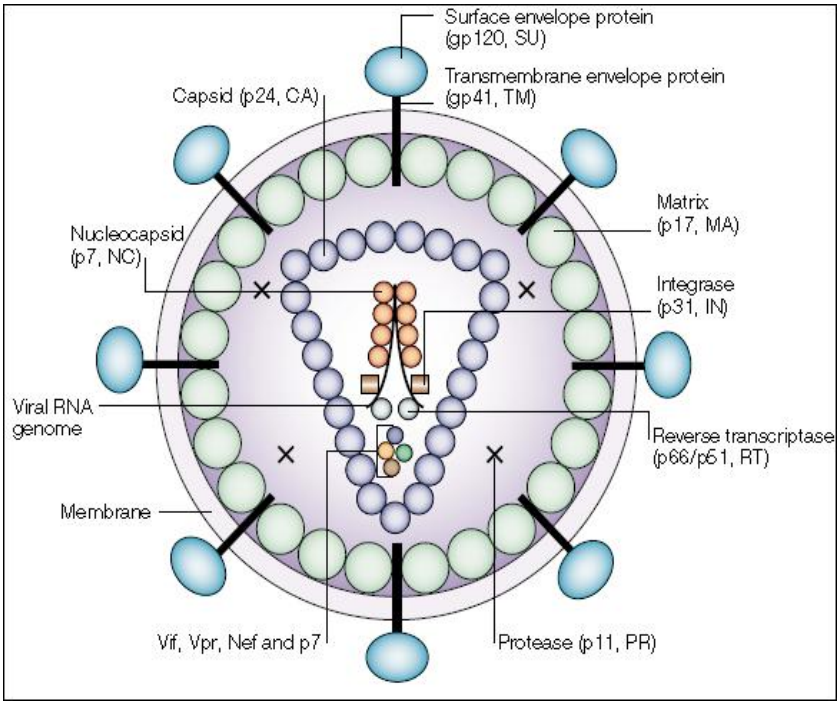


Figure 3: Diagrammatic representation structure of HIV-1 virus (Source: Robinson et al ,2002) (50)

The functional viral envelope spike exists as a trimer of gp120/gp41 on the virus surface(51). Structural data is available for the gp41 ectodomain in its fusogenic state, revealing a six-helix bundle consisting of a central parallel trimeric coiled-coil of the three N-HR helices, surrounded by the three C-HR helices in an anti-parallel hairpin fashion (52). Formation of the six-helix bundle is essential for fusion of the cellular and viral membranes. The surface

glycoprotein gp120 consists of five conserved (C1-C5) and five variable (V1-V5) regions(53). The V3 region was also found to protrude from the gp120 core, which could explain its immunodominant properties(54). CD4 binding was also observed to be dependent on sequences within the C3 and C4 regions (55)

Below the virus envelope is a layer of trimeric MA proteins which interacts with the lipid bilayer via amino (N)-terminal myristoyl groups. The MA shell surrounds a cone-shaped core, consisting of hexameric CA proteins forming a hexagonal lattice(56)(51). The conical core contains the nucleocapsid (NC) protein, which is closely associated with the viral genomic RNA. The NC protein is a small, 55 amino acid residue-protein, which contains zinc-finger motifs common to many proteins that bind nucleic acids (57)

2.7. HIV-1 genome

HIV has a diploid, linear, single-stranded RNA (9.5 kb genome) of positive polarity, which use a virus-encoded reverse transcriptase to convert their genomic RNA into DNA positive-sense. The DNA is subsequently incorporated into the host genomic DNA where it resides as a provirus, and consists of three main open reading frames, the gag (group-specific antigen), pol (polymerase) and env (envelope) genes (58). Sequencing of the HIV-1 genome in 1985 classified the virus as part of the Lentivirus genus ,whose other members include the sheep visna/maedi lentivirus that also cause slow disease syndromes in mammals (59).

The gag gene encodes a 55 kDa Gag precursor polyprotein (Pr55Gag), which is cleaved by the viral protease into matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7) and p6 proteins, as well as the two smaller spacer peptides SP1 and SP2 . The pol gene encodes the viral enzymes protease (PR or p15), reverse transcriptase (RT or p66 and p51) and integrase (IN or p31). These enzymes are initially synthesised as part of a Gag-Pol precursor polyprotein (Pr160Gag-Pol), which is produced by ribosome frameshifting near the 3’ end of

gag, but cleaved into individual enzymes by the viral protease(58).The env gene encodes an Env precursor glycoprotein(gp160), which is cleaved and processed by cellular enzymes to produce a non-covalent complex of a surface glycoprotein (SU or gp120) and a transmembrane glycoprotein (TM or gp41)(60)

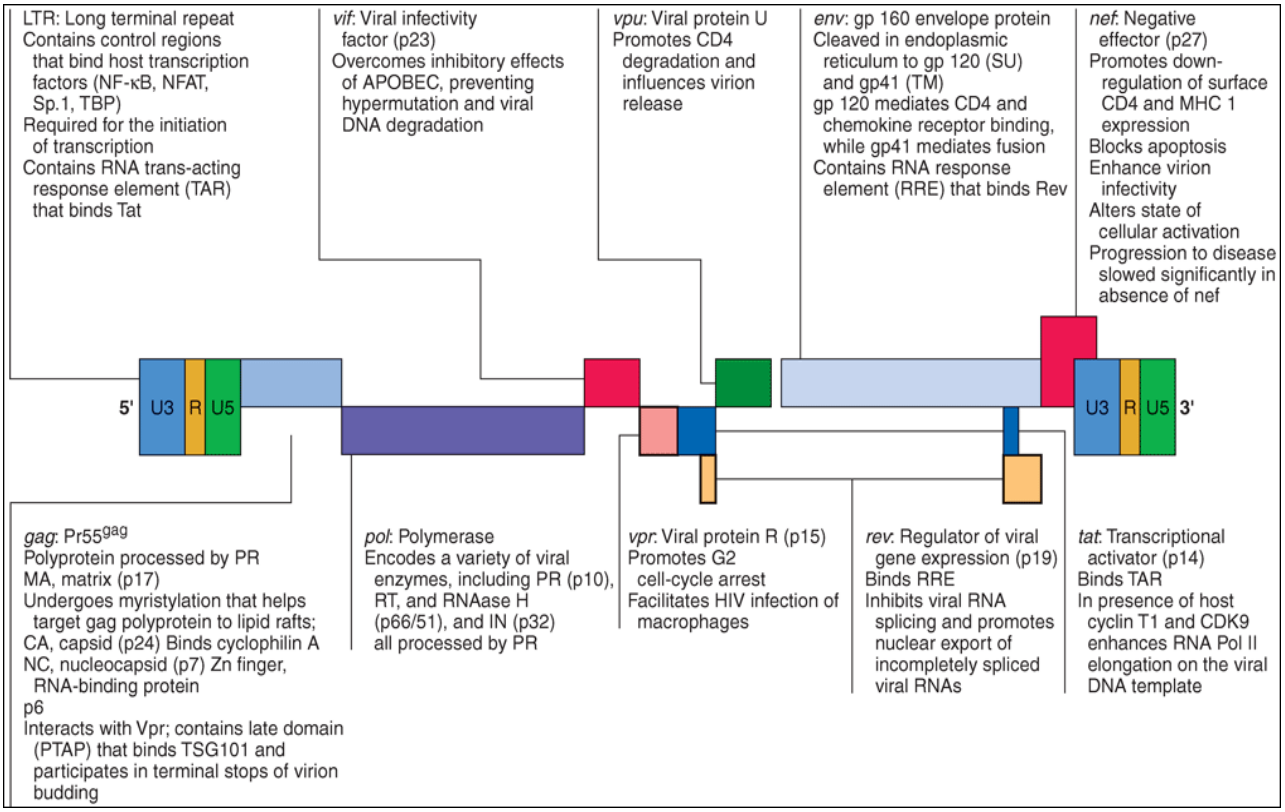


Figure 4: HIV-1 genome (source: Harrison's principle of Internal Medicine 18th edition)(5)

The HIV-1 genome also contains genes encoding regulatory and accessory proteins, located downstream of the pol gene(57). The tat and rev genes contain two exons each and encode the gene regulatory proteins Tat (Transactivator of transcription) and Rev (regulator of virion), whereas the vif, vpr vpu and nef genes encode the accessory proteins Vif (viral infectivity factor), Vpr (viral protein R), Vpu (viral protein U) and Nef (negative factor)(61) .The HIV-1 genome is flanked by two long terminal repeats (LTRs) at both the 5' and 3' end of the integrated provirus genome, and are 630-640 bp long(62). The LTRs consist of the U3 region that contains the viral promoter and enhancer sequences, the R region that contains the

polyadenylation signal, and the transactivation response element (TAR) that serves as the binding site for the viral Tat protein. The diagrammatic representation of HIV-1 genome and its component genes are summarized in the figure 4.

The secondary structure of the complete single-stranded RNA genome of HIV-1 was recently determined, which can function to regulate the translation and facilitate the proper folding of proteins with implications for viral fitness (63).

2.8. HIV-1 life cycle and replication

Study of viral replication helps us for the better understanding of viral activity and selection of therapeutic strategies for the control of virus. Stages are

2.8.1. Virus entry

2.8.1.1 .HIV-1 Receptors

Understanding of the cellular receptors for HIV-1 is crucial as the virus needs to interact with these receptors in order to gain entry into the host cell. Blocking of these interactions, for example through the use of antibodies, can potentially block virus infection of the cell. The cellular receptor for HIV-1 was identified to be CD4 in 1984(64), when they found that monoclonal antibodies (mAbs) to CD4 were able to block HIV-1 infection. The CD4 antigen is a transmembrane glycoprotein belonging to the immunoglobulin superfamily of receptors and is expressed predominantly on T helper cells but to a lesser extent on other cells types such as monocytes, macrophages, dendritic cells, eosinophils and mast cells(65). CD4 on T helper cells acts as a coreceptor by binding to the non-polymorphic regions of MHC class II molecules on antigen-presenting cells and is involved in the activation of antigen-driven T cell responses(66). In the initiation of the HIV-1 entry process, the gp120 portion of the envelope spike was discovered to bind to the D1 domain only(67).

As non-human cells engineered to express CD4 only were not susceptible to infection and other human components or cofactors are involved, It was realised that a coreceptor is required for HIV-1 entry(68). This missing coreceptor for HIV-1 entry was identified to be CXCR4 in 1996, where Feng *et al* . showed that co-expression of both CD4 and CXCR4 are required to render previously non-permissive cells susceptible to infection (69). However, this was found to support the infection of T cell line-tropic isolates of HIV-1 and not with macrophage-tropic strains and primary T-cell tropic strains, indicating that yet another co-factor was responsible for mediating entry for the latter isolates.

The coreceptor for macrophage-tropic HIV-1 was identified by several groups to be CCR5, which is also the receptor for various chemokines such as RANTES, MIP-1α and MIP-1ß(70). Other coreceptors, such as CCR2 and CCR3, can also mediate entry of some HIV-1 strains.The gp120 portion of the envelope spike was found to bind to the first and third extracellular domains of CCR5 and CXCR4 (54). All primary HIV-1 isolates use one or both of CCR5 and CXCR4 as co-receptor.

Virus isolates are now classified based on coreceptor usage, and are referred to as CCR5- or CXCR4-using viruses, or R5 and X4 viruses, or R5X4 for dualtropic viruses(71). CCR5 using viruses were observed to be preferentially transmitted over CXCR4 using viruses although CXCR4 using viruses have been associated with lower CD4+ T cell counts and faster progression to AIDS(72).

The HIV-1 entry process is initiated by attachment of the gp120 subunit of the viral envelope spike to the primary cellular receptor CD4(64). Binding of gp120 to CD4 triggers a series of conformational rearrangements in gp120 and in the envelope spike leading to the formation of a pre-hairpin intermediate which later springs out to insert the gp41 fusion peptide into the host cell membrane. This results in the exposure of the coreceptor binding site, thus allowing

either of the main cellular co-receptors CCR5 or CXCR4 to bind to gp120. The fusion of viral and cellular membranes coincides with formation of six-helix bundle(52). The V3 loop, in particular the amount of net positive charges on the V3 loop, is the main determinant for co-receptor usage and thereby cell tropism , although sequence changes in the V1, V2, C3 and C4 regions of gp120 and in gp41 have been implicated(54)(73).

Virus entry can also take place through cell-cell transfer via a virological synapse and are all dependent on HIV-1 envelope glycoprotein and CD4. Such cell-cell spread of virus and its ability to evade antibody responses remain unclear. Although the virus is generally thought to enter a cell through fusion with the plasma membrane, the virus has also been observed to enter via endocytosis in a pH-independent step(74).

2.8.2. Uncoating and Reverse transcription

After virus entry, the process of viral core uncoating which is unclear and formation of a reverse transcription complex (RTC) occurs. Uncoating involves the disassembly of CA and release of the viral ribonucleoprotein complex. This process remains to be elucidated but has been suggested to involve both viral and cellular factors (75), in particular the cellular protein cyclophilin A(57). The cellular tripartite motif protein TRIM5α can associate with the CA and promote its degradation, leading to accelerated core disassembly and uncoating, and thus restrict retroviral infection in a species-specific manner (Stremlau et al ., 2004;Stremlau et al , 2006).

The various TRIM proteins associated with inhibition of HIV-1 replication is summarized in table 1 below.

Table 1: Summary of TRIM proteins involved inhibition of HIV-1 replication.

Protein	Organism	Virus target	Replication step	Site	Ref
TRIM-5α	African green monkey, macaque	CA	Pre-RT	Cytoplasm	(76)
TRIM-5-CypA	Owl monkey	CA	Pre-RT	Cytoplasm	(77)
TRIM-19	Humans	?	Trafficking	Cytoplasm	(78)
TRIM-22	Humans	?	Transcription	Nucleus	(79)
TRIM-32	Humans	Tat	?	Nucleus	(80)

The RTCs are large nucleoprotein structures consisting of packed filaments containing IN and Vpr and interacts with the cytoskeleton(81). Reverse transcription of the viral genomic RNA into a double-stranded DNA genome is thought to take place essentially within the RTC by the viral RT, which possess both DNA polymerase and RNase H activities to degrade the genomic RNA template(82). The RT polymerase lacks any proof-reading ability and is therefore highly error-prone, making approximately 3.4×10^5 errors per base pair per cycle to cause the extreme sequence variation observed among HIV-1 isolates. In addition, RT binds with low affinity to its template and can hence make frequent jumps between the two RNA genomic molecules. If the two genomic RNA molecules are different, this ability results in the generation of genetically recombinant DNA genomes, thus contributing to genetic variability(83).

The reverse transcription process can be inhibited by a cellular cytidine deaminase protein called APOBEC3G, and thus restrict retroviral replication by deaminating cytosine residues to uridine. This causes gaunosine to adenosine hypermutation in the opposite strand hence

inactivates the viral replication. Sheehy et al described in HIV-1, the restriction overcome by the viral virion infectivity factor (Vif) protein, which mediates polyubiquitylation and proteasomal degradation of APOBEC3G, thus preventing APOBEC3G incorporation into virions and its modulation of the reverse transcription process(84).

2.8.3. Integration and transcription

HIV-1 replication requires that the virus enters the nucleus. Once the reverse transcription is complete, the RTCs are gradually transformed into pre-integration complexes (PICs) in a process that is little understood. The PICs contain the viral genomic DNA and viral protein IN, and are actively imported into the nucleus with the help of importin 7 (81), and the viral protein Vpr which contains nuclear localisation signals .Once the pre-integration complex has been imported into the nucleus, the viral DNA is integrated into the cellular genomic DNA and resides as a provirus in the host genome. This is solely mediated by viral IN and shown to specifically integrate into transcriptionally active regions of the host genome(85). Once integrated into the host genome, the provirus can stay latent or be transcribed by the cellular machinery. Prior to the production of the viral transactivator Tat, the HIV-1 promotor on the 5'LTR is activated by cellular transcription factors alone, resulting in low transcriptional levels and short transcripts. Once Tat is produced, it strongly enhances transcriptional activation and elongation in a positive feedback loop (86).

After transcription, the viral RNAs are polyadenylated, spliced and exported from the nucleus. The obtained transcripts are either unspliced (9 kb), incompletely spliced (around 4 kb), or fully spliced RNAs (around 2 kb). The primary HIV-1 RNA transcript or unspliced RNAs are used for expression of Gag and Gag-Pol precursors or packaged into virions as genomic RNA. Incompletely spliced mRNAs have the *gag-pol* region spliced out and can be used for expression of Env, Vif, Vpr and Vpu, whereas fully spliced mRNAs have the *gag*,

pol and most of *env* removed, contain no intron sequences, and are used to express Tat, Rev and Nef. The viral Rev protein promotes the nuclear export of unspliced and incompletely spliced viral RNAs by binding to a highly structured RNA region called the Rev responsive element (RRE).

2.8.4. Synthesis, assembly, and processing of viral proteins

The Gag (Pr55Gag) and Gag-Pol (Pr160Gag-Pol) polyproteins are synthesized on polyribosomes in the cytoplasm, and are important for membrane targeting and binding, multimerisation of Gag, encapsidation of viral genomic RNA and association with viral envelope glycoproteins anchored to the plasma membrane for

particle budding and release (58). The MA protein is important for membrane targeting and binding(56). Gag multimerization is mediated by the viral NC protein at the plasma membrane and a layer of Gag-particles associated with the inner layer of the plasma membrane that will eventually form a spherical immature virus particle, with the encapsidation of viral genomic RNA by NC(82).

The HIV-1 envelope glycoprotein, gp160, is first synthesized as a precursor polyprotein on the rough endoplasmatic reticulum (ER) where it undergoes glycosylation, folding and oligomerisation(86) which are made up of asparagine-X-serine/threonine motifs where X is any amino acid except proline. Proper folding of gp160 is mediated by the isomerisation of disulphide bonds and the signal peptide then cleaved. The gp160 also undergoes oligomerisation before exiting the ER and into the Golgi. Although dimers and tetramers have been observed, the functional spike exists as a trimer (67)(51)(86). To prevent the premature interaction of gp160 with CD4 in the ER, the viral protein Vpu has been shown to mediate CD4 degradation through ubiquitin-mediated proteolysis. CD4 cell-surface

expression is also down-regulated by the viral protein Nef, which mediates internalization and degradation of CD4 via clathrin-coated pits and lysosomes (87).The oligomeric gp160 is then transferred from the ER to the Golgi, where the newly added N-linked high-mannose glycans are modified by mannosidase enzymes, initiating the formation of complex N-linked carbohydrate glycans. The variability in glycosylation and processing of glycans contributes to the heterogeneity of the HIV-1 envelope. The trimeric gp160 then undergoes endoproteolytic cleavage in the Golgi to form the surface glycoprotein gp120 and the transmembrane glycoprotein gp41 by cellular serine proteinases. Cleavage occurs at a highly conserved lysine/arginine-X-lysine/arginine-arginine motif, After cleavage, gp120 and gp41 associate non-covalently with each other(88).The envelope spikes are then directed to the plasma membrane through the secretory pathway and incorporated into virions(58).

2.8.5. Assembly and budding of virions

Virus assembly and budding is generally believed to occur at the plasma membrane of infected cells. In macrophages, HIV-1 assembly occurs in late endosomes or intracellular multivesicular bodies. In T cells HIV-1 buds at the plasma membrane(89).The viral protein p6 is crucial for the release of virus particles, as the deletion of p6 has been shown to cause accumulation of assembled virus particles tethered to the plasma membrane . A recently described protein, tetherin, was found to tether virus particles to the cell surface, but was antagonised by the presence of the viral Vpu protein which was previously known to enhance virus release(90).

HIV-1 buds from the membrane of infected cells in an immature, non-infectious form .Upon budding, the viral PR is activated and cleaves the Gag precursor Pr55Gag in a stepwise fashion into the MA, CA, NC and p6 proteins.PR also cleaves the Gag-Pol polyprotein Pr160Gag-Pol, generating PR, RT and IN. Cleavage of the Gag and Gag-Pol polyproteins

leads to structural rearrangement of the individual Gag proteins and the formation of a mature, infectious HIV-1 virion containing the characteristic conical core(58).

The stages of replication of HIV-1 virus and the proteins affecting replication is summarized in figure 5.

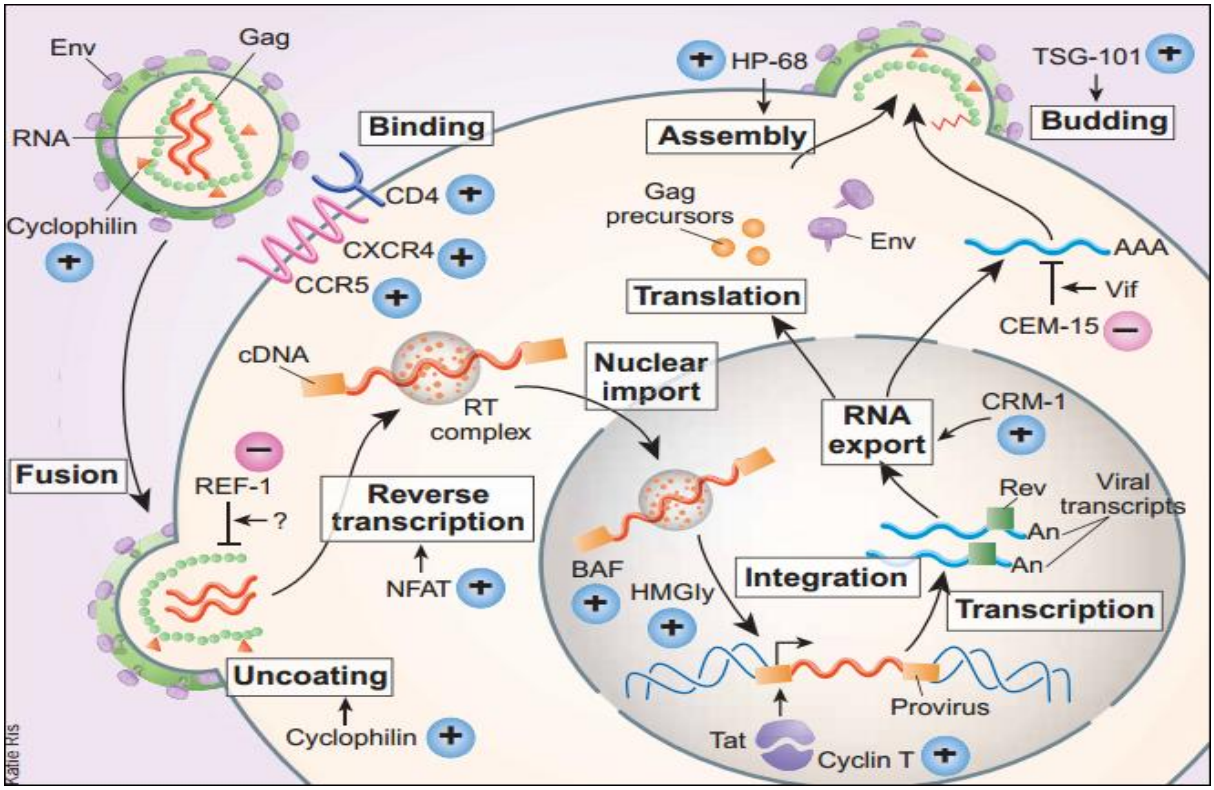


Figure 5: Stages of HIV-1 replication and the cellular factors that promote or inhibit the HIV-1 replication (Source: Stevenson et al ,2003) (91)

2.9. HIV-1 pathogenesis

The pathogenesis of HIV-1 is complex and multifactorial involving the interplay between multiple viral and host factors. The direct interaction between the viral envelope and its cellular receptor, CD4 along with either C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4), results in a scenario where the virus infects key cells of the adaptive immune response, and hijacks the host immune system. Following infection, a variety of intracellular mechanisms involving the host immunological factors and viral

regulatory and accessory proteins are important for the clinical course of disease progression. A significant disparity is observed in the disease course of HIV-infected individuals. While, those who succumb to AIDS relatively soon after infection are termed as rapid progressors and there are others, termed as long-term non-progressors who manage to evade clinical progression without therapy even after 20-25 years (5). Even more interesting, a group of patients named elite controllers can control the viremia below 50 copies/ml viral load without any signs of immunodeficiency.

2.9.1. Immune response in HIV infection

2.9.1.1. Innate and Humoral immunity

Mannose Binding Lectins and complement, are important soluble anti-HIV innate immune factors as they can inactivate virus. The effects of Tat is inhibited by anti-Tat IgM antibodies, and antileukocyte autoantibodies (IgM) could prevent HIV entry into cells(92).

Neutralizing antibodies against the HIV envelope gp120 and gp41 are one of the important adaptive immune responses. Antibodies to cell surface proteins like Lymphocyte Functional Associated molecule, Intracellular cell adhesion molecule (ICAM), human leukocyte antigen (HLA) can also mediate these antibodies. Antibodies that attach to virus-infected cells (via gp120 or gp41) mediate direct killing of infected cells through antibody-directed cellular cytotoxicity (ADCC) in which Fc-receptor of NK cell plays a primary role(92).

2.9.1.2. CELLULAR IMMUNITY

2.9.1.2.1. T-cell immunity in HIV

During the first weeks of infection, initial HIV-1–specific CD8+ T cell responses are induced. HIV-1 permanently escapes recognition by CD8+ T cell responses in the host due to continuous recombination and mutations in HIV-1(93). This plays a crucial role in the acute

phase of HIV-1 infection where the virus is not diversified yet. In the chronic phrase of infection the virus is more diversified.

2.9.1.2.2. CD4 T cell response

CD4+ T cell response in acute HIV-1 infection plays a crucial role in control of viral replication, and viral escape from CD4+ T cell–targeted epitopes. It is unclear whether the presence of HIV-1–specific CD4+ T cells is the cause of low viremia(94).

During primary HIV-1 infection, there is a massive infection of both resting and activated CD4+ T cells in gut-associated lymphoid tissue. This leads to destruction of up to 60% of these cells in the early days after infection. During primary HIV-1 infection HIV-1–specific CD4+ T cell responses is either simultaneously or earlier than CD8+ T cell responses. (95).
The role of other CD4+ T cell subsets and their role in the control of HIV-1 replication are also controversial. T helper 17 (Th17) cells causes immune activation, which has no beneficial effect in HIV-1 infection. Similarly, the role of HIV-1–specific Th2 or T follicular helper cell responses, which provide important helper signals for the maturation and antibody generation of B and plasma cells(93).
CD4+ T cells have a major role in helping the immune response of B cells and other T cells through cytokines. Some CD4+ cells exhibit cytotoxic activity. CD4+ T-cell support is particularly important for the efficient function of CD8+ T-cell immunity.T-cell coproduction of IL-2 and IFN-γ appears to be beneficial for anti-HIV immunity. The CD4+ T-cells and dendritic cells interaction plays an important role in HIV replication and production of specific cytokines as we. Strong HIV specific CD4+ cell responses alone and particularly in association with HIV-specific CD8+ T-cells provide a good prognosis for the clinical

course(97). The mechanisms of CD4+ T-cell destruction are summarized in the table 2 below.

Table 2: Mechanisms of CD4 T cell destruction (5)(96)

Direct	Indirect
• Loss of plasma membrane integrity due to viral budding	• Aberrant intracellular signaling events (Antibody Dependent Cell-Mediated Cytotoxicity)
• Accumulation of unintegrated viral DNA	• Autoimmunity
• Interference with cellular RNA processing	• Bystander killing of viral gp120–coated cells(Nef medicated FasL activation)
• Intracellular gp120-CD4 autofusion events	• Apoptosis(tat protein mediated caspase-8 upregulation)
• Syncytia formation(cyclin-dependent kinase-1 pathway)	• Inhibition of lymphopoiesis
• Vpr-induced G2 arrest and apoptosis	• Activation-induced cell death(Bcl-2 and TNF)
• Envelope mediated apoptosis	• Elimination of HIV-infected cells by cytotoxic T cells and NK cells

2.9.1.2.3. CD8+ T cell

CD8+ T-cells can function in both the innate and adaptive immune systems. The CD8+ cell antiviral factor (CAF), blocks HIV-1 transcription by not causing any destruction of the infected cell. CNAR/CAF appears to be an innate immune activity that differs therefore from the conventional adaptive cytotoxic CD8+ CTL antiviral response that kills HIV-infected cells expressing specific viral epitopes. CNAR is found highest in long-term survivors (LTS); when this activity decreases, virus replication resumes with progression to disease(98).

CD8+ T cell responses in primary HIV-1 infection are induced better when compared to CD8+ T cells generated under persistent viral infection with abundance of antigen. Naive CD8+ T cells mature into effector T-cells by recognizing the antigen and kills the respective target cells. Only a minor fraction of the effector cells develop into a memory cells.

In chronic persistent infections CD8+ T cells are exhausted due to persistent antigen stimulation(99). Increased IL-10 plasma levels in chronic HIV-1 infection have been demonstrated and suggested to contribute to the general dysfunction of CD8+ T cell responses(100).

2.9.1.2.3. Dendritic cells

DCs are play an important role in immunity. DCs act as a link between the innate and adaptive immune responses. The co-infection with gram-negative bacteria along with HIV-1 infection may facilitate HIV-1 spread by enhancing LPS-stimulated maturation of DC and, therefore, DC-mediated HIV-1 transmission to CD4+ T cells. IFN-α inhibits the cell-to-cell transmission of HIV-1 between CD4+ T cells and DC-mediated HIV-1 transmission to CD4+ T cells(101).

Matured DCs has got important role in the prevention of replication and spread of HIV-1. Capturing of the HIV-1 form the exposed mucosa is by the attachment of the virus to the CD4 and co-receptors which are expressed in low level in DC. A C-type lectin DC-SIGN expressed on DCs act as an adhesion molecule towards this. HIV-1 binding to DC-SIGN on the DC surface triggers a signaling cascade that promotes HIV-1 replication in DCs(102).

NK cells also have an important function in the pathogenesis of HIV and are influenced by the production of cytokines such as IFNs and IL-12. The interaction of NK-inhibiting receptors (KIRs) with HLA components prevents this activity. The enhanced expression of

KIRs, in the presence of HIV viremia, can suppress NK cell function. Recent study showed that NK KIR 3DL1 and its BW4-801 ligand are associated with better clinical outcome(103).

2.9.2. Cytokines in HIV

An array of interleukins has been studied in HIV patients on ART and its role in immunological response has been documented. Interleukin-2 (IL-2) is produced by activated T-lymphocytes that have a key role in triggering immune responses. The main effect of IL-2 is to induce the clonal expansion of T-lymphocytes after antigen recognition. The impairment in IL-2 production has been the first functional defect described in HIV-positive patients. Immune-based therapy with IL-2 when used as adjunctive therapy may further improve immune responses, demonstrated by an increase in CD4+ T-lymphocyte counts in recent clinical trials(104). IL-7 is cytokine produced by stromal cells, Bone marrow, thymus and lymph node and is critical for T-cell thymopoiesis. Elevated levels of serum or plasma IL-7 have been observed in CD4 T-cell lymphocytopenia, including HIV infection. Rajasuriar et al ,(2012) demonstrated a significant association between IL-7Ra haplotype 2 and faster CD4 T-cell recovery in Caucasians but there was no significant association in Africans(105). IL-10 is produced mainly by Th2 cells and occasionally by activated macrophages and non-hematopoietic cells (eg, keratinocytes)and administration of IL-10 to HIV-1-positive patients has also been shown to decrease the number of circulating HIV-1 virions. IL-10 inhibition of T-cell apoptosis could actually be beneficial for HIV-1-infected individuals. IL-10-producing B cells are induced early in HIV-1 infection, can be HIV-1 specific, and are able to inhibit effective anti-HIV-1 T cell responses(100). Sailer et al , in their recent study, have shown that, during the early stages of HIV-1 infection, interleukin (IL)–18 might suppress HIV-1 by increasing the Th1 immune response and reducing CXCR4 co-receptor expression(106).

IL-21 is induced during acute and chronic HIV-infection and correlates with relative control of virus. IL -21 producing HIV specific T-cells correlate to better control of plasma viremia (107).

2.9.3. IFN-λ

IFN-λ is a newly discovered family with similar mechanism of Type I IFNs. IFN-λ subfamily comprised of three structurally related cytokines (IFN-λ1, IFN-λ2, IFN-λ3), which are also called interleukin-29 and interleukin-28A/B (IL-29, IL-28A, IL-28B) respectively. IFN-λ is highly conserved in human populations, implying strong evolutionary selection for these genes for protection against infections (8).

The IFN-λ genes consists of six exons, which are each encoded by a single exon (7).The IFN-λs exert their activity through receptor made up of two subunits: IL-28Rα and IL-10Rβ (7). The IL-10Rβ subunit is expressed on several cell types with highly expressed in cells of epithelial origin(108).

High levels of IFNLs are secreted during viral infection of lung and liver especially in airway epithelial cells by respiratory viruses. IFN-λ are induced by many cell types, including plasmacytoid dendritic cells (pDCs), conventional dendritic cells, peritoneal macrophages, T cells, B cells, eosinophils, hepatocytes, neuronal cells, and epithelial cells, after virus infections or after activation of TLR3, TLR4, TLR7, TLR9, stimulation of RIG-I, or Ku70(109). IFN-λs are induced by either IFN regulatory factor 3 (IRF3), IRF7, or NF-kB pathways (110).

The IFN-λs bind as monomers to the IFN-λR (IL-28Rα), which then pairs with IL-10Rb to form the functional heterodimer receptor. IFN-λR signals are transmitted through the JAK1/TyK2, STAT1, STAT2, STAT3,STAT5, and IRF9 pathways to induce transcription of IFN-stimulated genes via ISGF3(110). These signals result in the induction of 2'-

5’oligoadenylate synthetase, serine/threonine protein kinase (PKR), ISG56, and IFN-λ2/3. By comparison with IFN-αß signals, IFN-λR induces longer lived activated(tyrosine-phosphorylated) STAT1 and STAT2, and more strongly induces IFN responsive genes (MX-1, ISG15, TRAIL,SOCS1). The cytoplasmic receptor RIG-I stimulation by viral RNA activates IFN-λ expression (111).The pathway mediated by IFN-λ is described in figure 6.

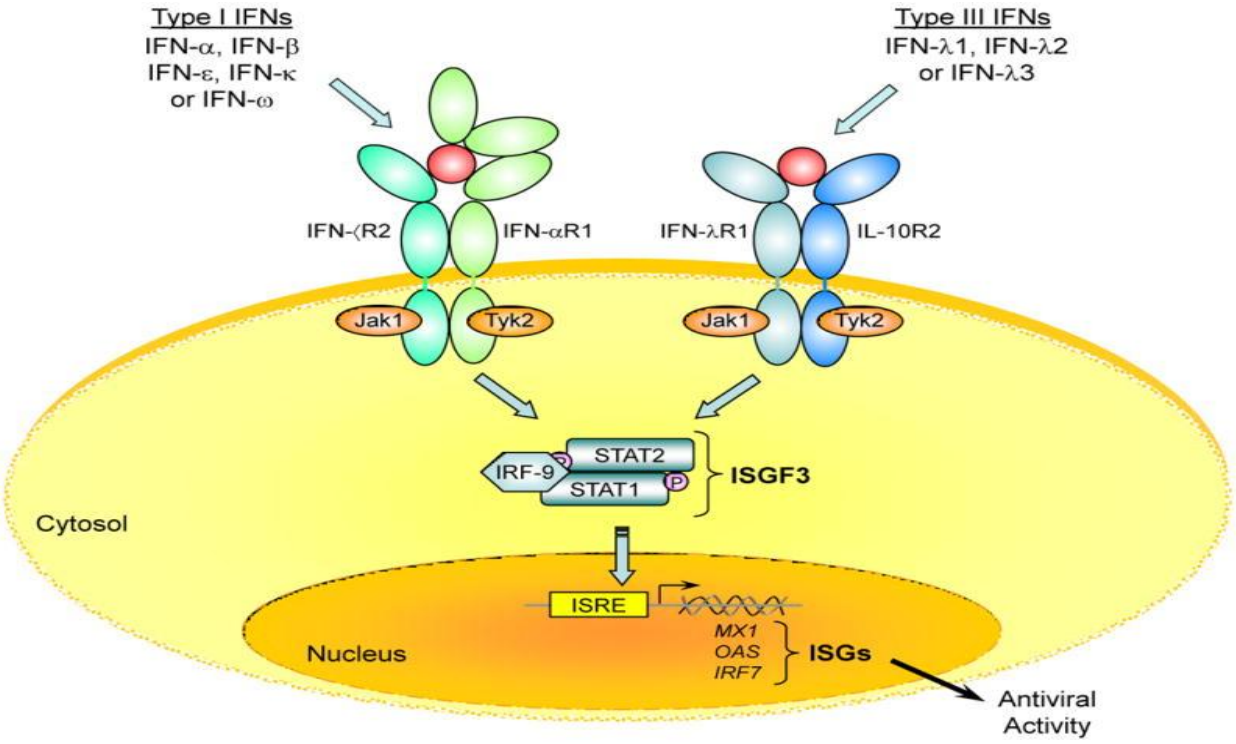


Figure 6: Signaling pathway of IFN-α and IFN-λ(Source: Donelly et al ,2011) (112)

Baseline ISG expression is an indicator of successful IFN-α treatment of HCV. Higher baseline hepatocyte ISG expression was observed in patients with chronic HCV infection and IFN-α nonrespnders. The SNPs in the IFNλ3/4 and IL-28RA genes found to have an association with elevated basal ISG expression and treatment failure (113). Important antiviral ISGs are ISG15, MX1, OAS1-3, and PKR . IFN-λ blocks the replication of numerous viruses in vitro, including encephalomyocarditis virus, West Nile virus, vaccinia virus, vesicular stomatitis virus, foot and mouth disease, HSV-1, influenza A virus, HIV (6),HCV, and hepatitis B virus.

2.9.3.1. Role of IFN-λ in HCV

The limit in level of IFN-λ induced in a natural HCV infection is unclear, as mechanisms are involved to inhibit the IFN-α/ß response in infected hepatocytes. The HCV NS3/4A protease inhibits IRF-3 activation and cleaves the RIG-I and TLR signaling adapters IPS-1 and TRIF. A NS2 protein blocks activation of IFN-λ through an unknown mechanism that is distinct from that of NS3/4A, and the HCV NS5A protein can also inhibit IFN-α/ß expression(114).. Furthermore, like IFN-α/ß, IFN-λ is expressed in PBMC but not in the liver of chronic HCV patients(115). Hepatitis C virus (HCV) NS3/4A, the viral protease responsible for cleavage of the viral polyprotein, can also cleave the key adaptor protein MAVS also named VISA, Cardif, or IPS-1 which transmits signals from upstream sensor molecules RIG-I or MDA5 to downstream TBK-1/IKKε and IKKα/β/γ complex. HCV NS3/4A can also cleave TRIF, key adaptor protein responsible for signal transmission ofTLR3 (116). This leads to blocking of both RIG-I- and TLR3-mediated activation of type I IFNs. HCV NS2 protein can also inhibit IFN-α, IFN-β, IL-29, and chemokine gene promoter activity(114).

2.9.3.2. IFN-λ in HIV

Studies explaining the role of IFN-λ IN HIV infected individuals are limited.

Liu et al(2006) , showed that IFN-λ3 inhibits HIV infection of macrophages through TLR3 and JAK-STAT pathway(117)

Serra et al(2008) , reported the expression of the CD4, CXCR4, and CCR5 genes was increased when the peripheral blood mononuclear cells were pretreated with IFNλ-2 and was found out that it was associated with enhanced HIV-1 binding and replication(118)

Hou et al(2009) , showed that IFN-λ enhances APOBEC3G and APOBEC3F expression at both the mRNA and protein levels in macrophage(6). RR Tian et al(2012) , showed that IFN-λ3 inhibits the HIV integration and transcription *in vitro* (119).

2.9.3.3. Role of IFN-λ in innate immunity and T- and B-cell modulation

2.9.3.3.1. Macrophages

The M0 phenotype of macrophage differentiates into M1 macrophage in response to IFN-γ. M1 macrophages produce high amounts of IL-12 and IL-23.It also produces low amounts of IL-10, IL-1, TNF-α and IL-6 leading to induction of Th1 cells.

In the other side M2 cells are induced by IL-4 or IL-13. M2 cells do not present antigen to T cells and produce minimal amounts of pro-inflammatory cytokines. The induction of Th2 cytokines and subsequent stimulation of T regulatory cells is the key function of M2 cells. The expression of IL-28RA on human DCs has been mostly seen on plasmacytoid DCs. They are potent producers of IFN-λ.

2.9.4.3. Role of IFN-λ in Th1 or Th2 balance

Virus-specific Th1 and cytotoxic T cells play a crucial role in control viral replication. On the other side B cells receive important growth signals from Th2 cytokines. Antigen presenting cells in particular dendritic cells and macrophages are the decision makers in differentiation of T-cell subsets into a Th1 or Th2 phenotype. IL-12 and IFN-γ are the critical for the differentiation of Th1 cells(120). Following the activation of Th1 cells through pathogen detection via pattern recognition receptors, antigen-presenting cells in particular DCs and macrophages secrete IL-12 in large amounts. IL-12 induces natural killer cells to produce further IFN-γ. The Th1 cells are induced mainly by transcription factor T-bet.

IL-4 and IL-2 are critical for T-helper cell (Th2) differentiation. The major transcription factor involved in Th2 lineage differentiation includes the IL-4-induced STAT6.This up-regulates the expression of GATA3.The effects expression of T-bet and GATA3 are opposing. The effect of IFN-λ in T-cell mediation is summarized in figure.

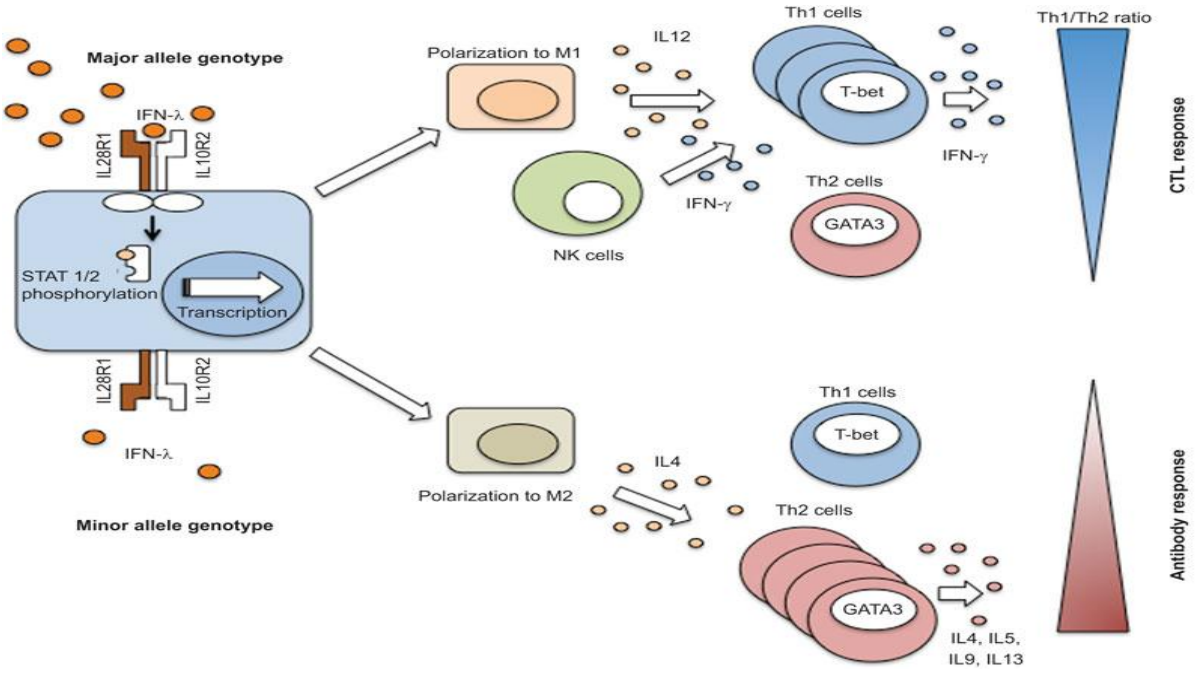


Figure 7:Impact of IFNλ3 SNP on cytokine profiling in viral infections(Source: Egli et al 2014)(121)

It is shown that IL-28RA which are responsive to IFN-λ is expressed in purified naive and memory human CD4 T cells. The expression of Th2 cytokines (IL-4 and IL-13) was suppressed in CD4 T cells by IFNλ1. GATA3 expression is reduced by IFNλ1 treatment. This suggests that the IFNλ1 has suppressing action on Th1/Th2 balance(122)

2.10. Host factor associated with HIV disease progression

2.11. HLA association with disease progression

The rate of disease progression and the different human leukocyte antigen (HLA) class I alleles are strongly associated, the interaction between the T cell receptor of the CD8+ T cells

and the HLA of the antigen-presenting cell is a key element in the overall control of HIV-1 replication(93).The association of various HLA with the HIV disease progression is summarized in table 3.

Table 3: HLA association with HIV disease progression

Fast progressors	Slow progressors / long-term survivors	High-risk exposed HIV-seronegative individuals
• HLA class I homozygosity • Ancestral haplotypes 8.1,35.1,44.2, A23	• Supertype A2	• A2
• Supertype B7, B*08	• BM27, BM51, BM57, BM1503	• DR13
• Haplotype A*01-B*08-R3,B22,B*35-Px, DR3, DR11	• Supertype DR13, Haplotype DRB1M13-QB1M06	

Other host factors associated with the disease progression are summarized in the table 4.

Table 4: Various Cellular genes affecting HIV disease progression(123)

Gene	Genotype	Effect on progression
Cell surface Coreceptors		
• **CCR5**	*+/Δ32*	Delay AIDS
• **CCR2b**	*+/64I*	Delay AIDS
HLA		
• **KIR/HLA**	*KIR3DL1/HLA-B*57*	Delay AIDS
• **KIR/HLA**	*KIR3DS1/HLA-B Bw4-80 Ile*	Delay AIDS

Cytokines		
• **IL-10**	*-592A*	Accelerate AIDS
• **IFN-γ**	*-179G/T*	Accelerate AIDS
• **IL-4**	IL-41-589T	Delay AIDS
• **RANTES**	*In 1.1C haplotype*	Accelerate AIDS
• **RANTES**	*-403A/-28G*	Delay AIDS
• **MIP-1α**	-	Delay progression
MBL	Homozygosity-lower MBL levels	Accelerate AIDS; enhance infection
APOBEC-3G	*186R*	Accelerate AIDS
TSG101	HapC	Accelerate AIDS
TSG101	HapB	Delay AIDS

2.11. Genome wide association studies (GWAS)

Several studies have been conducted to study the influence of host genetic factors in HIV infection. Genome wide association studies related to susceptibility, transmission, disease progression (AIDS) with HIV infection are summarized in the table 5.

Table 5: Summary of GWAS conducted associated with HIV.

Cohort	Year	Phenotype	Most important association	Validation	Ethnicity	Ref
CHAVI	2007	RNA VL set-point	HCP5(rs2395029)	Significant, confirmed	Caucasian	(124)
			-35 HLA-C(rs9264942)	Significant, confirmed		
		CD4 decline	ZNRDI(rs9261174)	Confirmed		
ANRS PRIMO	2008	Plasma HIV RNA in primary infection	HCP5(rs2395029)	Significant, confirmed	Caucasian	(125)
		Cellular HIV DNA primary	HCP5(rs2395029)	Significant, confirmed		

		infection				
GRIV	2009	Long-term non-progression	HCP5(rs2395029) C6orf48(rs9368699)	Significant, confirmed	Caucasian	(126)
Euro-CHAVI, MACS	2009	RNA VL setpoint	HCP5(rs2395029) -35 HLA-C(rs9264942)	Significant, confirmed	Caucasian	(127)
GRIV	2009	Rapid progression	PRMT6(rs4118325) SOX5 (rs1522232)	Putative	Caucasian	(128)
International HIV controllers study	2010	VL controllers	>300 SNPs in MHC −35 HLA-C (rs9264942) HCP5 (rs2395029) MICA (rs4418214) PSORSIC3 (rs3131018)	Significant Significant, confirmed Significant, confirmed Significant Significant	Caucasian African Hispanic	(129)
Queen Elizabeth Central Hospital, Malawi	2010	Mother-to-child transmission	HS3ST3A1 (rs8069770)	Putative	African	(130)
MACS	2010	Progression to AIDS	PROX1 (rs17762192)	Confirmed	Caucasian	(131)
DoD HIV NHS and MACS	2010	RNAViral Load setpoint	HLA-B*5703	Significant, confirmed	African	(132)
GRIV,MACS,ACS	2010	Long-term nonprogression (VL >100copies/ml)	CXCR6 (rs2234358)	Significant, confirmed	Caucasian	(133)
CHAVI, Malawi	2011	HIV acquisition	rs1946518(IL-18)	Significant	African	(134)
MACS	2011	Progression of AIDS 1987	PARD3B (rs11884476)	Significant, confirmed	Caucasian Africa	(135)
Blood donors, Sanquin	2011	*in vitro* HIV-1 replication in	DYRK1A	Putative	Caucasian	(136)

Amsterdam		macrophages	(rs12483205)			
ACS	2011	progression to AIDS,or AIDS-related death	AGR3 (rs152363)	Putative	Caucasian	(137)
Thailand	2011	Nevaripine tolerance	CCHCR1 (rs1265112)	Significant	Asian African	(138)
African serodiscordant couples cohort	2011	HIV acquisition	Nil	Not significant	African	(139)
Multicenter Hemophilia Cohort Study (MHCS)	2013	Resistance to HIV	CCR5Δ32 Heterozygosity CCR5Δ32 Homozygosity	Not significant	Caucasian	(140)
AIDS Clinical Trials Group A5095	2014	CXCR4 coreceptor usage and HIV disease progression	CCR5 Δ32(rs333) CCR5 Δ32(rs1799987) SDF1-3′A	Not significant	Caucasian Hispanic African American	(141)
Malawi	2014	Nevirapine hyper-sensitivity	HLA-C*04:01	Significant	African	(142)
CHAVI014	2014	Resistance to HIV	HLA/KIR	Not significant	Caucasian	(103)
ALIVE, MACS, SFCC, MHCS	2014	Host resistance to HIV acquisition CD4+ T cell depletion	ZNRD1 (rs3132130)	Significant	African-American, Caucasian	(143)

2.12. IL-28B polymorphism

In 2009, four landmark studies described a clinical association between IL28B and HCV RNA clearance with pegylated interferon and ribavirin combination therapy. This was associated with SNPs found in the *IL28B* gene locus, that encodes for IFN-λ3(144)(8). The SNP at rs12979860 SNP is associated with approximately 2-fold differences in spontaneous clearance and response to treatment(144). The wild genotype is associated with better outcomes, and heterozygous T/T genotype, showed the worse outcomes. The SNp at rs8099917 SNP also shown to be associated with a 2-3 fold difference in spontaneous clearance and response to therapy(9). The location of IL-28B genes and SNPs are depicted in Figure 8.

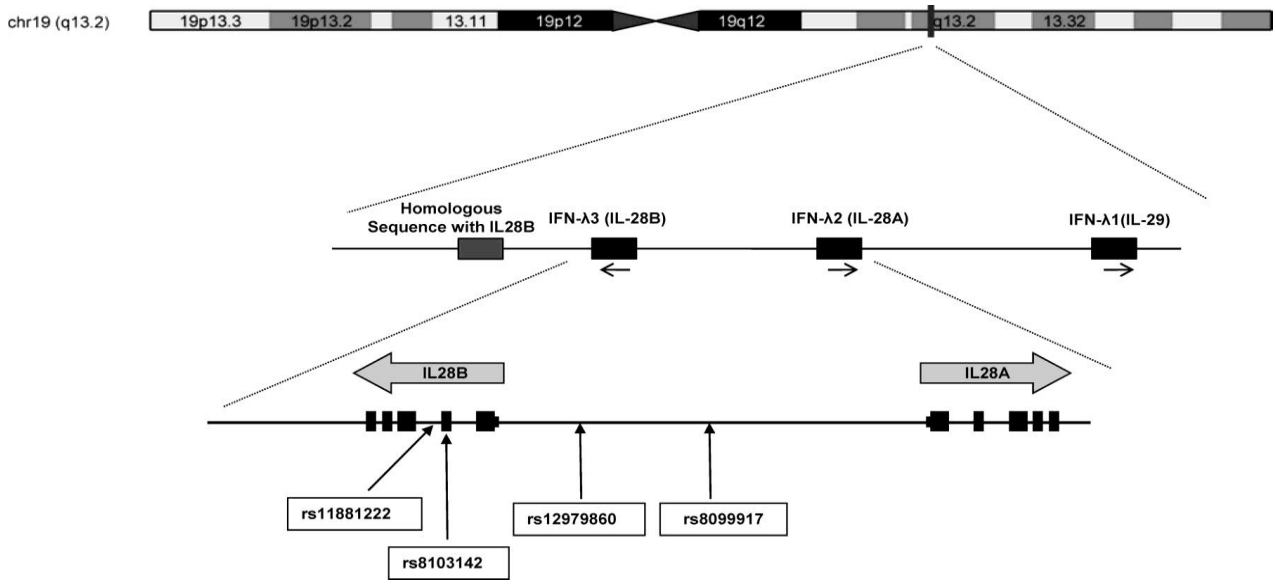


Figure 8: Location of interferon lambda genes and the SNPs (rs12979860 & rs8099917) associated with IL-28B chromosome 19(Source: Ito K et al ,2014)(145)

The polymorphisms associated with poor response to therapy are found at a higher frequency in African populations compared to European populations, consistent with the lower response rates of PEG-IFNα plus ribavirin treatment in African-Americans. Individuals harboring the rs8099917 minor allele were found to have reduced IFN-λ3 expression levels in PBMC, indicating that this variant may be located within a transcriptional regulatory element (8).

Because IFN-α upregulates IFN-λ expression, IFN-λ may amplify interferon-stimulated gene expression following administration of PEG-IFN-α in HCV infected individuals. As other host factors have also been associated with therapy outcome, the interplay between these various factors needs to be better defined. For example, patients who fail to achieve a sustained viral response after PEG-IFN-α therapy have a high pre-therapy level of intrahepatic ISG expression(146).

2.12.1. Mechanism of IL28B polymorphism in HCV

Langhans et al described the suppression of IL29 levels in patients with chronic HCV infection(147). The unfavorable rs12979860 genotype was associated with suppressed serum levels of IL29 and IL28A/B and this suppression appeared to be caused by HCV E2 and NS3 proteins(147).This indicates that IL29 levels are suppressed in chronic HCV infection, especially in patients with IL28B genotypes associated with poor response to treatment. Hence the lack of IFN-λ may present is responsible for non response. IFN-λ can directly inhibit HCV replication in vitro via signalling through the JAK-STAT pathway(115).Furthermore, liver and peripheral blood mononuclear cells (PBMCs) from patients with chronic HCV infection seem to express more IL28A/B RNA than do healthy controls(148).

IL28B rs8099917 has been associated with differences in IL28B expression with the protective allele leading to an increase in messenger RNA (mRNA) expression in healthy individuals(8). Langhans et al also reported higher IL29 levels in patients with the rs12979860 protective genotype as compared to the nonprotective genotypes . In contrast, Abe et al reported low IL28B levels in liver samples of patients with the rs8099917 protective genotype . In general, IFN-stimulated gene (ISG) induction was reported to below

in rs8099917 and rs12979860 protective genotypes(149). The presence of an active HCV RdRp in the cytoplasm generates signals to stimulate IL28B expression and that transcription at the IL28B promoter is influenced by the alleles present at rs28416813 which is in close proximity with IL28B gene(150).

2.12.2. IL28B Polymorphism in HIV

Studies describing the effects of IFN-λ on susceptibility of HIV-1 infection, replication and its association with ART are limited.

Study by Sajadi et al , 2011 a cohort study in HIV-1 Natural Viral Suppressors(NVS) concluded that IL28B CC genotype does not account for the noted HIV control in NVS cohort.

Martin *et al* (2010) showed that IL28B rs12979860 does not influence the susceptibility to HBV or HIV infection or the progression of HIV infection(151).

Rallon *et al* (2011) examined the protective role of IL28B polymorphisms in 29 seronegative individuals at risk for HIV-infection and in 68 HIV-positive carriers with and without rapid progression of immunodeficiency. No protective role of IL28B polymorphism was found examining both HIV-disease progression and HIV-protection(11).

Interestingly, HTLV-1 infected individuals with the CT/TT allelic variants at the IL28B rs12979860 gene exhibited approximately a 3-fold increased risk of HAM/TSP and had nearly 10-fold higher median HTLV-1 proviral loads (PvLs) than CC carriers(152)

Kamihira et al . reported that the frequency of the rs8099917, located 3 kb upstream the IL28B/IFN-λ3 gene had no significant association with susceptibility to HTLV-1 infection or the development of Adult T-cell Leukemia in the Japanese subjects(153).

2.12.3. Impact of IL28B genotype on IFNλ3 secretion

The C/C genotype leads to higher levels of IFNλ3 secretion in HCV infected patients. In addition to its anti-viral activity, IFNλ3 exerts potent immunomodulatory effects allogeneic stem-cell transplantation patients carrying the T/T genotype produce less IFNλ3(154), which in turn results in lower levels of circulating Tregs, leading to a more efficient expansion of CMV-specific T cells, which are critically involved in controlling CMV replication in either the presence or absence of antiviral therapy, the impact of the donor IL28B SNP genotype would be more pronounced than that of the recipient provided that IFNλ3 is mostly produced in response to defined stimuli by macrophages and pDCs, which are of donor origin(110).

If there is any association of CC or TT genotype with HIV susceptibility and progression that can be used as a prognostic marker in HIV-1 infected individuals

2.12.4. Activation of IFN-λ

A series of SNPs of IFNL3 and IFNL4 genes have been discovered and that impact the binding of transcription factors and methylation sites. This affects the the promoter output.(144)(113).

Reduced IFNλ3 expression during chronic HCV infection in liver biopsies, serum and PBMCs were reported with SNPs rs12979860 and rs8099917 (146). SNPs in rs12979860 were also associated with lower IFNλ3 gene expression in CMV infected fibroblasts and stimulated PBMCs (155).Data on the role of IFNLs and host response to virus are widely available with HCV infection that has been classically treated with (pegylated) IFN-α. This lead to a robust antiviral immune response in patients with chronic HCV infection. There is a difference in the expression kinetics between different IFNL family members during

stimulation of fibroblasts and human peripheral blood mononuclear cells (PBMCs)(155). IFNλ3 shows a peak at 24-h after stimulation with CMV, while IFNλ1 peaked by 6 hours by measuring mRNA levels(155). The effect of IL-28B polymorphism on IL-28B expression is summarized in table 6.

Table 6: Impact of IL28B polymorphism on IL28B expression reported among different viral infections

Gene	SNP	Genotypes	Impact on expression (minor allele)	Impact on virus replication and immunity(minor allele)	Ref
IFNλ3*	rs12979860	Major: C/C Minor: C/T,T/T	Reducing IFNλ3	**HCV:** lower rates of spontaneous clearance **Acute CMV:** reduced replication	(113) (156)
IFNλ3*	rs8099917	Major: T/T Minor: T/G,G/G	Reducing IFNλ3	**HCV:** lower rates of spontaneous clearance **Acute CMV**: high ISG production and reduced replication less priming of CMV-specific T cells	(113) (156)

***** Originally these SNPs were assigned to or close to IFNL3,recent studies show that true location is IFNL4 for rs12979860 and between IFNL4 and IFNL4P1 for rs8099917(157)

The C allele of IFNL3 is found to have better inhibition of the the initial replication of a virus. This is due to the long lasting anti-viral effect of induced ISGs. This will lead to the control or the clearance of the virus. Compared to this among the individuals with minor allele thee is low level of IFNL3 and reduced anti-viral and modulatory ISG induction. The limited IFN-α response lead to a high, short-lived ISG expression further causing replication of viruses and subsequent selection of quasi-species resistant to ISGs. The constant viral replication in with low IFNL3 mediated regulatory activity enhances the IFN-α-mediated ISG

expression. The impact on IFN-λ production in viral infection by major and minor genotypes is summarized in figure 9.

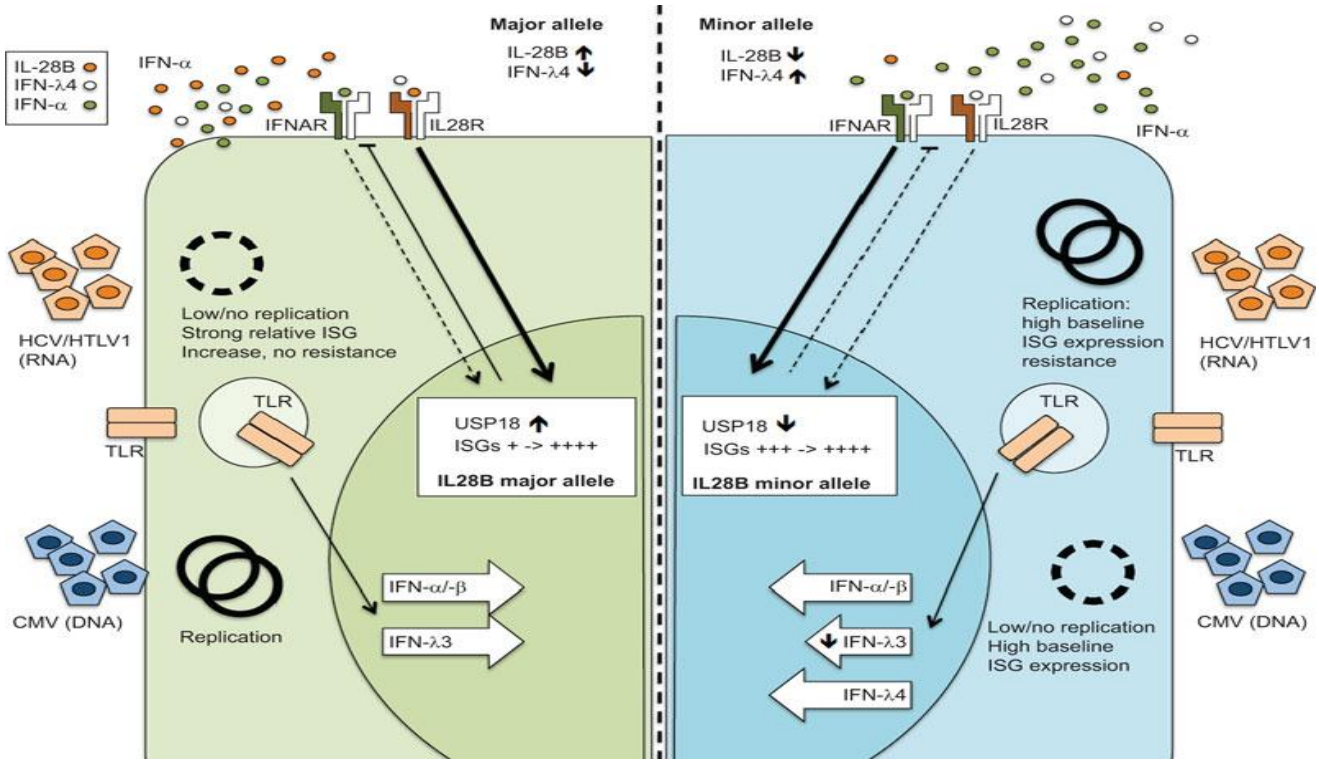


Figure 9: Summary of ILNλ3 SNP and impact on virus replication(Source: Egli et al 2014)(121).

Kamihira et al .2012 (153)showed that found a strange phenomenon that the IL- 28B mRNA expression levels in peripheral blood were lower in samples with HTLV-1/HCV co-infection than in samples with either HTLV-1 or HCV alone, especially significantly for HTLV-1 mono-infection. In particular, samples carrying TT homozygotes were strongly downregulated, more than the minor TG hetero- and GG homozygotes.

2.12.5. IFN feedback

The rs12979860 and rs8099917 SNPs in the IFNL3 gene region were associated with lower IFNλ3 expression levels and higher baseline ISG expression(144) .There is higher ISG expression in IFNλ3 SNPs in spite of lower IFNλ3 gene expression which can be due to

(i) A disruption in the regulatory aspects (at ligand or receptors) of type-I and -III IFN signaling cascades

(ii) Significantly lower IFNλ3, anti-viral and modulatory ISG induction observed among T/G allele IFNλ3 SNP.

2.12.6. Studies conducted in India

A study by Sivaprasad et al studied the distribution of genotype and allelic frequency of IL-28B gene polymorphism in healthy uninfected controls in Andhra Pradesh, India and it was found that the frequency (59%) of CC genotype (wild) is significantly higher than CT or TT in south Indian population.

Studies on association of IL-28B polymorphism with HCV and PEG-INFα/RBV treatment in Indian population are limited.

Chinnaswamy et al , showed that SNPs like rs12979860 and rs8099917 identified by GWA studies are in high Linkage Disequilibrium with rs28416813 and exert their effect on the phenotype through rs28416813.Strong linkage disequilibrium between rs28416813 and another important SNP rs12979860 in two ethnic populations was reported. Hence this study demonstrated that the SNPs at the IL28B locus influence spontaneous clearance and treatment response in chronic HCV infections(150).

Firdaus et al , concluded that CC, TT the two favourable markers at SNPs rs12979860 and rs8099917 are strongly associated with sustained virological response in genotype 3 infected population(10).

Gupta et al , concluded that the IL28B CT/TT genotype is strongly associated with the treatment non-response in patients infected with HCV genotype 3 and CC genotype of IL28B is associated with higher quick viral response (158).

Hence, IFNλ3/4 gene polymorphism and IL28RA and its modulation of IFNλ3 expression have an effect on Th1 and Th2 cytokine production and memory formation during viral infections. Hence more studies to be conducted for the better understanding and treatment interventions.

3. Clinical Stages in HIV infection

The infection and clinical progression of HIV-1 is generally divided into three phases:

a) Primary or acute infection

The first phase of HIV-1 infection is termed as primary infection and occurs a few weeks after infection. During this phase, a high viremia (acute phase viremia) due to active replication of virus is seen and the patient is highly infectious during this phase. The symptoms are generally non-specific and hence frequently not recognized as signs of HIV infection.

Due to the strong host immune defense during acute phase, there is a drop in viremia termed "set point". The risk of viral transmission between hosts largely depends on the viral set point(159). This phase is termed as latent phase since there are new or only minor clinical symptoms and typically varies between 5 to 15 years.

b) Assymptomatic phase

Following 6 months of acute infection phase, majority of the individuals become asymptomatic and usually the viral set point will be <20,000 RNA copies/ml. This is a reflection of the host antiviral responses by the innate and adaptive immune systems. Neutralizing antibodies, NK cell and HIV specific T cell activity also leads to the reduction in the HIV activity. In certain individuals even the viral load become undetectable they are called 'elite controllers'(160). HIV replication during the subsequent persistent infection

period can take place at low levels in the lymph nodes and other tissues and appears to reflect the extent of control by antiviral immune responses. In average most of the treatment naïve individuals after 10 years of asymptomatic phase start showing symptoms. There is a loss of immune activities especially HIV-specific CD4+ and CD8+ T-cell activities. Viral destruction of lymphoid tissue mirrors this progression of HIV infection(160).

c) AIDS

The last phase is AIDS which is characterized by rapid loss of CD4+ T-cells and highly elevated viral load. Due to the destruction of the body's immune mechanism, AIDS-associated opportunistic infections and malignancies are manifested during this phase(5).

The stages of AIDS progression with the association of viral load is summarized in figure10.

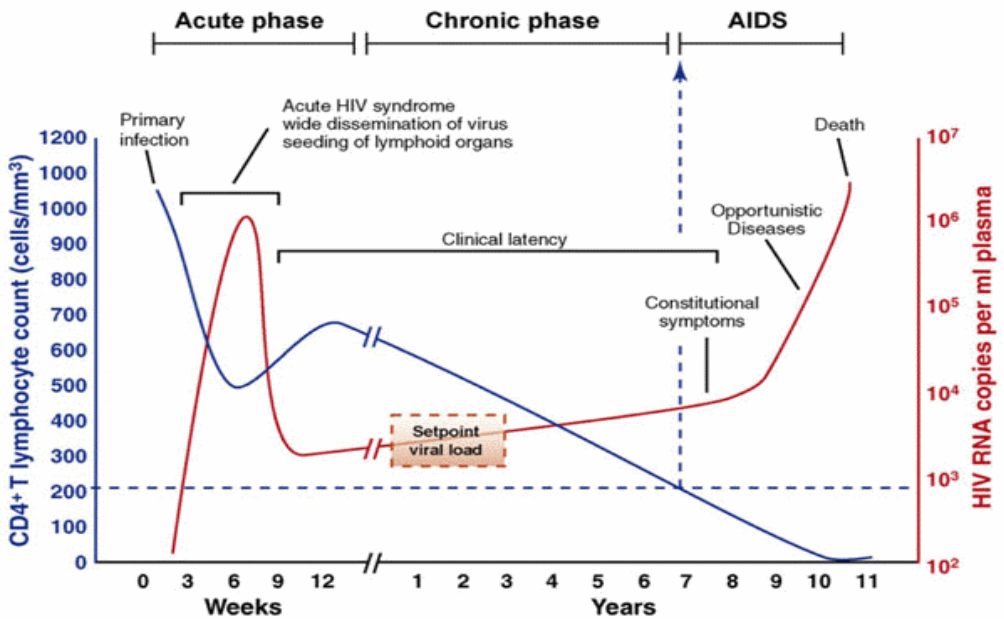


Figure 10:The stages of AIDS progression(Source: O'Brien et al 2013) (159)

3.1. Staging of disease:

There are two system of staging employed in HIV infection. One is the WHO staging system and the other is CDC staging. The most commonly used one in developing countries is WHO staging is described in table 7. WHO gives the clinical staging of HIV based on clinical manifestations and an independent staging based on immunological criteria based on the CD4+ T cell counts. These criteria apply to adults above the age of 15 years(42).

Table 7: WHO staging of HIV/AIDS

WHO staging of HIV/AIDS for adults and adolescents
Clinical stage 1
• **Asymptomatic**
• **Persistent generalized lymphadenopathy (PGL)**
Clinical stage 2
Herpes zoster
Angular cheilitis
Recurrent oral ulcerations
Papular pruritic eruptions
Seborrhoeic dermatitis
Fungal nail infections of fingers
Clinical stage 3
Oral candidiasis
Oral hairy leukoplakia
Pulmonary tuberculosis (TB) diagnosed in last two years
Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis
Conditions where confirmatory diagnostic testing is necessary

Laboratory confirmation required

Unexplained anaemia (<8 g/dl), and or neutropenia (<500/mm3) and or thrombocytopenia (<50 000/ mm3) for more than one month

Clinical stage 4

Conditions where a presumptive diagnosis can be made on the basis of clinical signs or simple investigations

HIV wasting syndrome

Pneumocystis pneumonia

Chronic herpes simplex infection (orolabial, genital or anorectal of >1month duration)

Oesophageal candidiasis

Extrapulmonary TB

Kaposi's sarcoma

Central nervous system (CNS) toxoplasmosis

HIV encephalopathy

Conditions where confirmatory diagnostic testing is necessary:

Extrapulmonary cryptococcosis including meningitis

Disseminated non-tuberculous mycobacteria infection

Cryptosporidiosis

Isosporiasis

Visceral herpes simplex infection

Cytomegalovirus (CMV) infection (retinitis or of an organ other than liver, spleen or lymph nodes)

Any disseminated mycosis (e.g. histoplasmosis, coccidiomycosis, penicilliosis)

3.4. IRIS

Immune Reconstitution Inflammatory Syndrome (IRIS) is a clinical entity described in HIV infected individuals following adminstation of HAART and measurable viral suppression can lead to acute worsening of symptoms and signs associated with underlying opportunistic infections (161). There is deterioration of clinical status following ART though there is improved immune function. This may be due the inflammatory response against the infectious antigen. Immune reconstitution inflammatory syndrome usually sets in patients with low CD4 (usually <50cell /µl) prior to treatment. Usually this sets in within 6 weeks of ART. One of the reason for IRIS may be the polymorphisms of gene encoding IL-6, IL-12, and TNF-α. Tadokera et al , 2013 has shown that of IL-10 family cytokines showed an increase in levels of transcript for IL-10 and IL-22 in IRIS patients compared to non-IRIS controls(162).

4. Antiretroviral Therapy

The primary goal of ART is to reduce HIV associated morbidity and mortality. ART will help in the durable viral suppression and the treatment improves immune function and lowers risk of both AIDS defining and non-AIDS-defining complications and prolongs life. Additional benefits of ART include reduction in HIV-associated inflammation and its associated complications.

4.1. Antiretroviral therapy in India

As on March 2013, there are around 18.13 lakhs People Living with HIV (PLHIV) registered at the 400 ART Centres functioning all around the country. Currently near 6.5 lakhs are on first line ART. Along with this 840 Link ART Centres primarily established for dispensing

ARV drugs, monitoring side effects and treating minor OIs. Among this 154 LACs have been upgraded as LAC plus

centres to provide Pre ART services additionally(3)

The drug regimens available in India as per NACO 2012 guidelines is summarized in the table 8.

Table 8: The ART Regimens provided to HIV infected individuals as per the NACO guideline (2012)

Regimen	Drugs	Comments
Regimen I	Zidovudine + Lamivudine + Nevirapine	Hb ≥9gm/dl, not with ATT
Regimen I(a)	Tenofovir + Lamivudine + Nevirapine	Hb <9 gm/dl, not with ATT
Regimen II	Zidovudine + Lamivudine + Efavirenz	Hb ≥9 gm/dl, with ATT
Regimen II (a)	Tenofovir + Lamivudine + Efavirenz	Hb <9gm/dl, with ATT, Hepatitis B and/or Hepatitis C co-infection, Pregnant women
Regimen III	Zidovudine + Lamivudine+ Atazanavir/ Ritonavir	Regimen I & III toxicity to both NVP and EFV. 2^{nd} line regimen- TDF containing first line regimen if Hb ≥ 9 gm/dl
Regimen III(a)	Zidovudine + Lamivudine + Lopinavir / Ritonavir	Regimen III with severe Atazanavir toxicity First line regimen for patients with HIV-2 infection with Hb ≥ 9 gm/dl
Regimen IV	Tenofovir +Lamivudine+ Atazanavir/ Ritonavir	Second line regimen for on AZT/d4T containing regimen in the first line. Patients on TDF containing first line

		regimen who develop toxicity to both NVP and EFV
Regimen IV (a)	Tenofovir + Lamivudine+ Lopinavir/Ritonavir	Regimen IV with severe Atazanavir toxicity 1st line for HIV 2 infection with Hb < 9 gm/dl First line for Women exposed to sd-NVP in the past
Regimen V	Stavudine+ Lamivudine+ Atazanavir/ Ritonavir	Second line for TDF containing regimen in the first line if Hb < 9 gm/dl
Regimen V(a)	Stavudine+Lamivudine+ Lopinavir/ Ritonavir	Regimen V with severe Atazanavir toxicity

5. HIV Vaccine

It is been 30 years since the advent of HIV, many vaccine trials were conducted. Table 9 shows the details of all the trails carried out.

Table 9: Vaccine trials conducted for HIV-1

Vaccine candidate	Phase	Components	Countries hosted	Ref
Canarypox plus(RV144) Envelope	3	gag,pro, env (E) plus gp120 (B, E)	Thailand (Sanofi Pasteur)	(163)
Ad5 (HVTN 502/ Merck 023)	2b	gag, pol, nef (B)	Dominican Republic, Haiti, Jamaica, Peru, South Africa,	(164)

			United States	
DNA plus Ad5 (HVTN 505)	2	gag, pol, nef (B), env (A, B, C) plus gag, pol (B), env (A, B, C)	Kenya, Haiti, Jamaica, Rwanda, South Africa, Tanzania, Uganda, United States	(165)
Canarypox plus lipopeptides	2	gag, pol, nef, env (B) plus cytotoxic T-lymphocyte epitopes (B)	France	(166)
DNA plus protein	1	T helper epitopes from gag, pol, vpr,nef (B)	Peru, United States	(167)
DNA plus peptides	1	gag (B) multiple T-cell epitopes(plus or minus IL-15 or IL-12 adjuvant or GM-CSF)	Brazil, Thailand, United States	(168)
DNA–PLG plus envelope	1	gag, env (B) plus oligomeric gp140 (B)	United States	(169)
Anthrax-derived polypeptide-HIV gag fusion protein	1	gag (B)	United States	(170)
DNA plus modified vaccinia Ankara	1	gag, pol, nef, tat, env (C)	United States	(171)
Modified vaccinia Ankara	1	gag, pol, nef, tat, env (C)	India	(172)
Fowlpox plus modified vaccinia Ankara	1	gag, pol, nef, tat, rev, env (B)	Brazil, United States	(173)
Adeno-associated virus	1	gag, pr, rt (C)	Belgium, Germany, India, South Africa, Zambia	(174)

Venezuelan equine encephalitis viral replicon	1	gag (C)	Botswana, South Africa, United States	(175)

The Thai phase 3 (RV144) vaccine regimen, ALVAC prime/bivalent clade B/E recombinant gp120 boost, provided an estimated 31.2% efficacy against the acquisition of HIV-1 infection at 42 months after vaccination. Multiple HIV-1 vaccine efficacy studies have been completed, yet only one trial in Thailand resulted in partial efficacy (RV144)(176). Another trial in Thailand (VAX003) that used a protein component of the RV144 vaccine showed no efficacy. The VAX003 clinical trial, in a high-risk injection drug use cohort, containing the same bivalent clade B/E gp120 protein immunogen as RV144, without the ALVAC prime did not show protection despite higher vaccine elicited neutralizing antibodies (nAbs) compared to RV144(176).

Hence alternative approach like enhancing cytokine level, modulating the T-cell immunity seems a better way of the control of viral replication and hence reduce AIDS associated complications and morbidity associated with HIV infection

There is no study documenting the association of IL28B polymorphism in HIV infected individuals on ART. Based on the studies of association of IL28B polymorphism and the better clinical outcomes and the anti-HCV properties of IL28B, in this study we looked at the effect of IL28B polymorphism on IL28B production and immunological recovery in HIV infected individuals on ART. If there is any association of CC or TT genotype with HIV susceptibility and progression that can be used as a prognostic marker in HIV-1 infected individuals and IL28B can be used as an adjuvant to Antiretroviral Therapy.

3. Materials and Methods

This study was conducted in the Department of Clinical Virology, Christian Medical College, Vellore between July 2013 and August 2014. Patients were recruited from whom attending the Infectious Diseases (Medicine I) and Dermatology and came to the virology department for CD4+ T cell testing and or HIV-1 viral load estimation.

a) Approval for the study

The approval for the study was obtained from the Institutional Review Board, CMC, Vellore (IRB Min No: 8237 dated 19.03.13).

b) Sample size

Though it is a pilot study the sample size was calculated using the EPI6 software based on a study on IL-28 polymorphism published from India. Taking into account the prevalence of the CT/TT genotype of 41% in healthy population(40) and precision of 14 with 5% α error the sample size was calculated as 48.

c) Inclusion criteria: i) Patients

1) Individuals with documented HIV infection by WHO-NACO strategy 3.

2) Age >18years

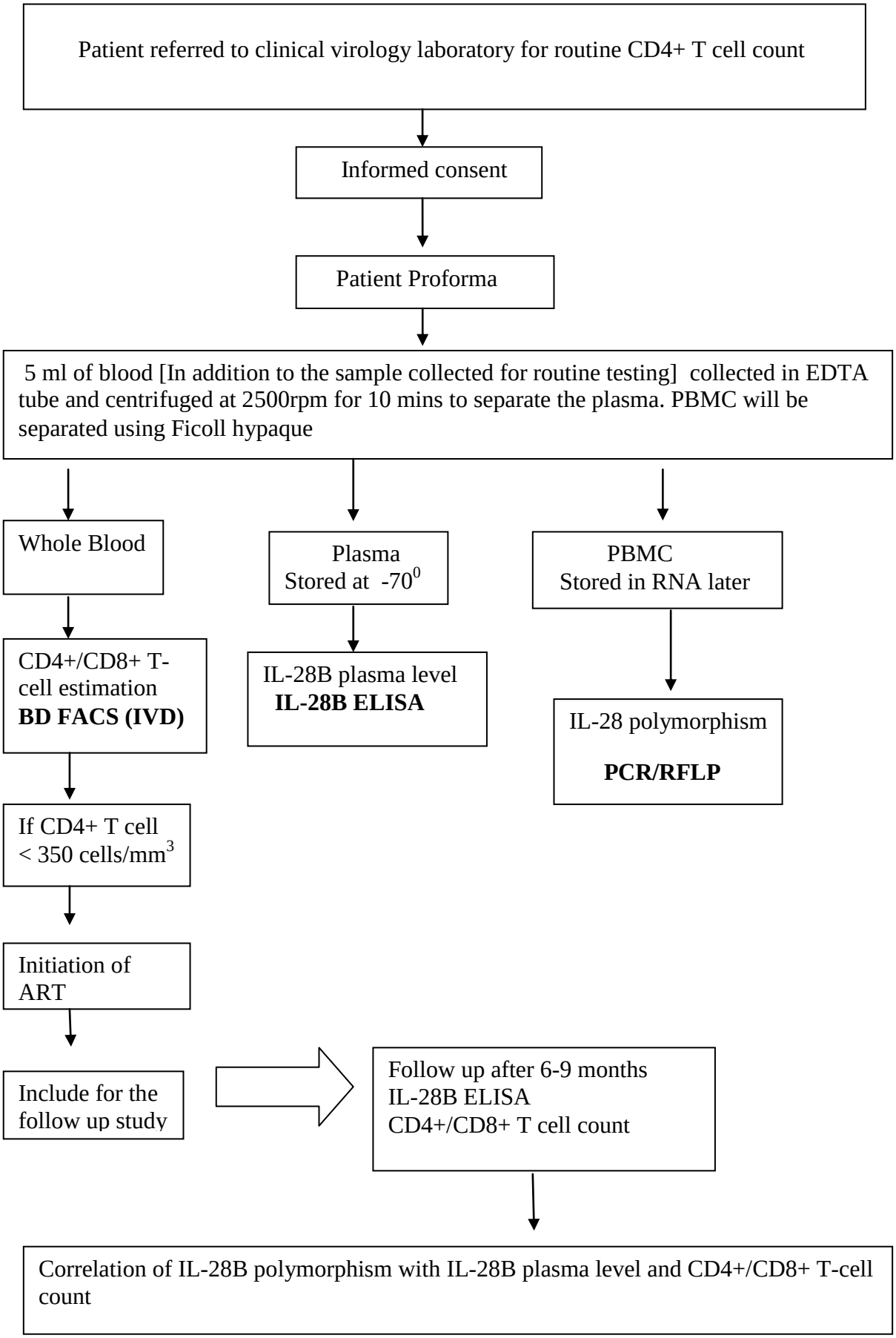
3) HIV-1 infected individuals not on ART with CD4 + T cell count less than 350 cells/µl [eligible to initiate ART]

4) Individuals who gave consent to be a part of the study

ii) Controls

Thirty age and sex matched HIV negative normal healthy individuals (for two cases one control was selected) who gave consent were also recruited for the study. Healthy age and sex matched laboratory staff or patient attendees (relatives) were recruited as controls after screening for HIV-1 infection using one of the NACO approved rapid test (PAREEKSHAK® HIV 1/ 2 Triline) according to WHO-NACO strategy III.

Algorithm of study:



3.1. CD4+/CD8+ T-cell count estimation by flow cytometry :

CD4+/CD8+ T cell counts were performed on blood samples collected using the BD FACS(IVD) kit by BD FACS count flow cytometer (Becton Dickinson, New Jersey, U.S.A)

i) Principle:

The assay employs a two colour immunofluorescence method to measure the absolute T lymphocyte count (cells/µl of whole blood) of CD3+T cells and CD3+CD4+ T cells, CD3+CD8+ T cells. The reagents for detection consist of a mixture of monoclonal antibodies conjugated with two fluorochromes and a known number of fluorochrome integrated polystyrene beads. The tubes contain reagents with antibodies to CD4/CD3 and CD8/CD3. When whole blood is added in the tubes with the reagents, the antibodies bind specifically to the CD antigens on the surface of the T cells. The instrument detects the fluorescent signal and measures the relative size of the cells.

ii) Procedure:

Fifty µl of whole blood (with EDTA) was added to the tubes provided with premeasured reagents containing fluorescent labeled antibodies and incubated for 60 minutes. 50µl of fixative is added to the sample in the tube and vortexed. Control bead sets containing fluorochrome integrated polystyrene beads in four levels (zero, low, medium, high) are included to validate the linearity of the assay. The samples are run in the instrument after the controls are passed. The software analyzes and gives the printed report of CD4, CD8, total average CD3 cells and CD4/CD8 ratio.

3.1. Quality control for CD4 count:

All the blood samples for CD4+T cell count were collected between 8.00 a.m to 11.00 a.m in our laboratory to avoid the diurnal variation in the count. With every run, one sample from

the previous day is included as the internal quality control (IQC) samples. The samples were stored at room temperature (between 20° and 28°C) and analyzed within 48 hours of sample collection. The IQC values of a given day are compared with the previous day's values of BD FACS CD4+/CD3+ counts and percent variation calculated. Percent variation is calculated as follows: (count on the first day/count on the second day) -1 ×100. A sample showing more than 20% variation from the previous day's value is considered not acceptable and the clinical samples are retested if necessary. In addition to internal quality control we also use commercial stabilized blood (BD Multi-Check control, Becton, Dickinson, San Jose, CA) as external quality control for routine quality control testing. Our laboratory is also a participating External Quality Assessment Scheme (EQAS) programs under NARI/NACO. Under this EQAS program, every year 2 batches of QC samples sent by National AIDS Research Institute, Pune, India (NARI)/NACO are estimated for their CD3, CD4 values using the BD FACS Count system. The results obtained using our system are sent to NARI for evaluation. Since participation we always obtained values in acceptable range and passed the QC every time.

3.2. Separation of PBMC using Ficoll- Paque :

PBMC was separated from the whole blood sample within 24 hours of sample collection using Ficoll-Paque plus (GE healthcare, Uppsala, Sweden).

PBMCs were separated from whole blood using Ficoll-Paque^{TM} PLUS (GE healthcare, Uppsala, Sweden). Anticoagulant-treated blood is layered on the Ficoll-Paque PLUS solution and centrifuged for a short period of time. Differential migration during centrifugation results in the formation of layers containing different cell types. The bottom layer contains erythrocytes which have been aggregated by the Ficoll and therefore, sediment completely through the Ficoll-Paque PLUS. The layer immediately above the erythrocyte layer contains

mostly granulocytes which at the osmotic pressure of the Ficoll-Paque PLUS solution attain a density great enough to migrate through the Ficoll-Paque PLUS layer. Because of their lower density, the lymphocytes were found at the interface between the plasma and the Ficoll-Paque PLUS with other slowly sedimenting particles (platelets and monocytes) as showed in figure 11.

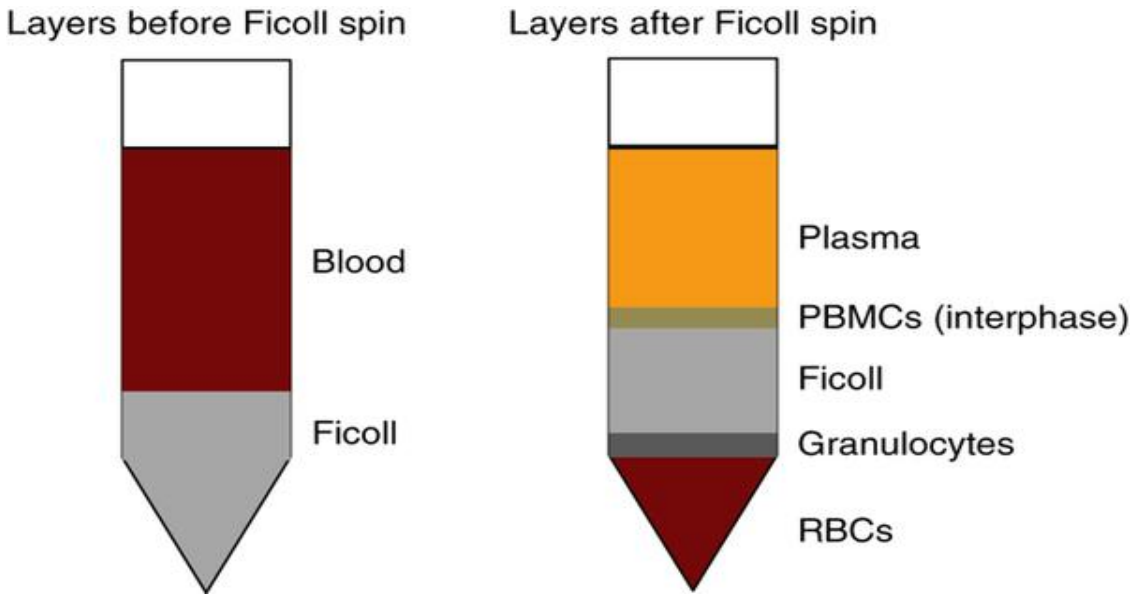


Figure11 : Layers formed after centrifuge using Ficoll Paque Plus(177)

The lymphocytes were then recovered from the interface and subjected to short washing steps with a balanced salt solution to remove any platelets, Ficoll-Paque PLUS and plasma. The cells were carefully retrieved. The PBMCs were stored in RNA LATER as aliquots of 100µl at concentration of $1x10^5$ cells at -80ºC in 1.7ml micro centrifuge tubes.

3.2.1. Procedure:

1. Label five centrifuge tubes with the sample number.

2. Mix the blood sample well and pipette 3ml of whole blood into the centrifuge tube. Dilute the blood with 6ml PBS. Mix well to dilute the sample.

3. Pipette out 3ml of Ficoll-Paque into 3 separate centrifuge tubes.

4. Carefully layer 3ml of the diluted blood sample onto the Ficoll-Paque in the three separate centrifuge tubes using the 1000µl reach pipette tips.

5. Centrifuge the centrifuge tubes at 2000 rpm for 30 minutes at 20ºC.

6. After centrifugation, three separate layers comprising of RBC at the bottom, Ficoll-Paque and plasma at the top are obtained. The PBMC are seen as an interphase between the Ficoll-Paque and plasma layer.

7. Carefully remove the plasma layer without disturbing the cells above the Ficoll-Paque. Aspirate the cells from all three centrifuge tubes and place them in a new centrifuge tube.

8. Centrifuge the aspirate at 2000rpm for 10 minutes.

9. Discard the supernatant. Add 5ml of PBS. Mix well and centrifuge at 2000rpm for 10 minutes.

10. Discard the supernatant. Add 1.5ml of PBS, mix well and centrifuge again at 2000rpm for 10 minutes.

11. Discard the supernatant. The washed cells are mixed in 400µl of PBS.

12. The cells are stored as $1x10^5$cells/ml aliquots of 200µl mixed with 200µl of RNA Later at -80ºC.

3.3. IL-28 polymorphism detection –PCR-RFLP

DNA extraction (Qiagen) from PBMC's was done using Qiamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions.

3.3.1.Procedure:

1. Add 20µl of Proteinase K to 200µl of the sample in a 1.5ml of micro centrifuge tube. Add 200µl of PBS with up to 2 x 10^5 lymphocytes.

2. Add 200µl of Buffer AL to the sample. Mix well by pulse-vortexing for 15 seconds.

3. Incubate the above mixture at 56ºC for 10 minutes in a dry bath.

4. Centrifuge the sample for a brief time to remove the moisture on the lids.

5. Add 200µl of 100% ethanol to the sample and mix well by pulse-vortexing for 15 seconds.

6. Centrifuge the sample to remove the moisture from the inside of the lids.

7. Transfer the above mixture to a Qiagen Mini spin column which is placed on a 2ml collection tube.

8. Centrifuge the Mini spin column at 8000rpm for one minute.

9. The flow through along with the collection tube is discarded and the mini spin column is placed on a fresh collection tube.

10. Add 500µl of buffer AW1 to the mini spin column and centrifuge the sample at 8000rpm for one minute.

11. Discard the collection tube with the flow through. Transfer the mini spin column onto a fresh collection tube.

12. Add 500µl of AW2 buffer to the spin column and centrifuge this at 14,000 rpm for 3 minutes.

13. Discard the collection tube with the flow through. Transfer the mini spin column onto a fresh collection tube and centrifuge at 14,000 rpm for 1 minute at 4ºC.

14. Discard the collection tube and place the mini spin column in a 1.5ml micro centrifuge tube. Add 200µl of elution buffer AE to the spin column and incubate at room temperature for one minute.

15. Centrifuge the above at 8000rpm for one minute.

3.3.2. Genomic DNA quantification

The extracted DNA was quantified spectrophotometrically using Take3, Gen5^{TM}, Biotek. 2 µl of the extracted DNA was loaded on the Take3 microplate and readings were taken using the Gen5^{TM} software. Both the concentration and purity of DNA was determined.

3.3.3. PCR

PCR amplification of rs12979860 and rs8099917 was performed using primers mentioned below in table 10:

Table 10: Sequence details of the primers used for IL-28B SNP PCR.

SNP	Primer Name	Sequence
rs12979860	rs60-F	5’ GCGGAAGGAGCAGTTGCGCT 3’
	rs60-R	5’ GTGCCTTCACGCTCCGAGCA 3’
rs8099917	rs17-F	5’ CCCACTTCTGGAACAAATCGTCCC 3’
	rs17-R	5’ TCTCCTCCCCAAGTCAGGCAACC 3’

Source: Sharafi et al , 2013 (178)

The lyophilized primers were reconstituted using Tris EDTA (TE) buffer and stored in aliquots at -20^{0}C.The standardization of the PCR was done using different primer concentration and annealing temperatures. The reaction volume and the cycling conditions PCR was standardized as mentioned in table 11 and 12.

Table 11: The volume of different reagents used in the PCR Reaction

Hotstar Taq Master Mix	**12.5(µL)**
Primer rs60f(10 pmol)	1(µL)
Primer rs60r(10 pmol)	1(µL)
DNA(100-300ng)	10.5(µL)
Total	25(µL)

3.3.3.1. Procedure:

1. Separate master mix was prepared for the appropriate number of reactions with the above template in Clean (PCR reagent preparation) room.

2. The master mix prepared was distributed to required number of 0.5ml PCR tubes.

3. DNA extracts were removed from the storage area, brought to room temperature and spun briefly in a micro-centrifuge.

4. 10.5 µl of DNA was added to make the final volume was 25 µl.

6. Amplification reactions were carried out in Veriti ^{TM} Thermal Cycler (Applied Biosystem, California, USA)

Table 12:The Cycling conditions used for the amplification of IL-28B gene.

rs12979860(^{o}C)	**rs8099917(^{o}C)**	**Time**	**Cycles**
95	95	15 minutes	1
95	95	30 seconds	40
60	56	30 seconds	40
72	72	45 seconds	40
72	72	7 minutes	1

Expected band size of the amplified products.

Post PCR amplification, the products are detected by Gel electrophoresis, the gel was visualized by ultraviolet radiation using Quantity one® (version 4.6.2) software in the gel documentation system (BioRad, California, USA).The expected band size are

rs12979860 – 241bp

rs8099917 – 552bp

Quality Control for IL28B SNP PCR:

a) Positive control: A preciously known extracted DNA is amplified, the expected band size are as follows.

rs12979860 – 241bp

rs8099917 – 552bp

b) Negative control: Milli Q water.

3.3.4. Restricted Fragment Length Polymorphism (RFLP)

The product of the PCR was used for RFLP analysis using Restriction Endonuclease BstUI and BsrDI (*New England Biolabs*®, UK). The reaction volume is as follows(table 13& table 14).

a) rs12979860

Table 13: The volume of different reagents used in the RFLP

Reagants	Volume per reaction(µl)
BstUI	2(µL)
Cut-smart Buffer	2(µL)
Nuclease free water	8(µL)
Amplified product	8(µL)
Total volume	20(µL)

b) rs8099917

Table 14: The volume of different reagents used in the RFLP

Reagants	Volume per reaction(µl)
BsrDI	2(µL)
NE Buffer	2(µL)
Nuclease free water	8(µL)
Amplified product	8(µL)
Total volume	20(µL)

The reactions for rs12979860 were incubated at 60°C for 15 minutes, and those for rs8099917 at 65°C for 15 mins Veriti ^{TM} Thermal Cycler (Applied Biosystem, California, USA).

3.3.4.1. Gel Documentation following RFLP

Ten microlitres of each amplicon was mixed with 2 µL of 6X loading dye bromophenol blue and then subjected to electrophoresis in freshly prepared 3% agarose gel containing 0.5 µg/ml ethidium bromide. Molecular ladder (Low range DNA Marker-A, Bio Basic Inc, Canada) used was 25-500 bp long. The gel was visualized by ultraviolet radiation using Quantity one® (version 4.6.2) software in the gel documentation system (BioRad, California, USA).The band size for genotypes are summarized in Table 15

Table 15: The expected bands of different genotypes are as follows

rs12979860(band size)	Genotype	rs8099917(band size)	Genotype
241	TT	**552**	TT
196,45	CC	**322, 230**	GG
241, 196, 45	CT	**552, 322, 230**	GT

Quality control for RFLP:

Positive control: Previously positive samples were included

rs12979860 – CT- bands at 241,196 and 45bp

rs8099917 – GT- bands at 552,320 and 232bp.

3.4. IL-28B plasma level estimation by ELISA:

IL-28 plasma level was estimated by a Human IL-28B in-house quantitative ELISA kit (R&D Systems, Minneapolis, MN, USA)[Catalog Number: DY5259] as per manufacturer's instructions.

Principle:

The system utilizes quantitative technique of "Sandwich" ELISA where the target protein (antigen) is bound in a "sandwich" format by the primary capture antibodies coated to each well-bottom and the secondary detection antibodies added subsequently by the investigator. The capture antibodies coated to the bottom of each well are specific for a particular epitope on the Human IL-28B cytokine, while the user-added detection antibodies bind to epitopes on the capture target protein.

3.4.1.1. Procedure for PBS preparation.

1. Dissolve the salts in distilled water and make up the volume to 1000ml as mentioned in table 16.

2. Adjust the pH to 7.4 with 1N NaOH.

3. Autoclave at 121ºC for 15 min at 15lb.

4. The autoclaved solution is stored at 4ºC. For every use PBS is diluted with sterile MilliQ water to a dilution that is one in tenth of the original concentration. The pH is checked and adjusted before every use.

3.4.1.Preparation of phosphate buffered saline(PBS)

Table 16: Materials used in preparation of PBS

Materials	Quantity
NaCl	8gm
KCl	0.2gm
NA2HPO4	1.15gm
KH2PO4	0.2gm
Distilled water	1000mL

Reagant Diluent: 1% Bovine Serum Albumin is added to 1x phosphate buffered saline. Reagant Diluent.

Wash Buffer: 0.05% Tween®20 in PBS, pH 7.2-7.4 in 1x phosphate buffered saline

3.4.2. Procedure for ELISA

Plate Preparation:

1) Capture Antibody (mouse anti-human IL-28B) is diluted to the working concentration of 2µg/mL in 1x Borate Buffer. Immediately coat a 96-well microplate with 100µL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at 4^0C.

2) Each well is aspirated and washed with 400µL of wash buffer three times using automated washer (Biotek, Winooski, USA) and blotted against clean paper towel.

3) 300µL of reagent diluent is added to each well for blocking and incubated at room temperature for a minimum of one hour.

4) Aspiration and wash step is repeated as in step 2

Assay procedure:

1) Standards (recombinant human IL-28B) reconstituted in reagent diluent containing recombinant human IL28B is diluted in seven 2-fold serial dilutions of with high standard being 2000pg/mL followed by 1000, 500, 250, 125, 62.5, 31.25 pg/mL was used.

2) 100µL of sample and standards in reagent diluent is added. The plate is covered with adhesive strip and incubated for 2 hours at room temperature.

3) After the aspiration/wash as in step 2 of plate preparation, 100µL of the Detection Antibody diluted in reagent diluent to working concentration of 500ng/mL is added to each well. Cover with a new adhesive strip and incubate for 2 hours at room temperature.

4) After the aspiration/wash as in step 2 of plate preparation, 100 µL of working concentration of Streptavidin-HRP(1:200) in reagent diluent was added to each well. Cover the plate and incubated for 20 minutes at room temperature avoiding the plate to direct light.

5) After the aspiration/wash step as in step 2 of plate preparation,100 µL of 1:1 mixture of Tetramethylbenzidine(TMB) and H2O2 is added to each well and incubated for 20 minutes at room temperature avoiding direct light.

6) 50µL of stop solution(2N H2SO4) is added to each well.

7) The optical density of each well is determined using microplate reader (Biotek®, Winooski, USA) by taking reading using dual wavelength of 450nm and 570nm.

Calculation of results

The average of concentration for each sample was calculated. Using standards in 7 dilutions and zero standard, a 4-Parameter logistic(4-PL) curve was generated with concentrations on X-axis

and Delta OD values on Y-axis using Gen5 software and concentration in each clinical sample was calculated based on Delta OD.

Statistical analysis

Continuous variables like CD4, CD8, CD3 counts and CD4/CD8 ratio were reported as mean ± standard deviation or median. Continuous variables of the two visits (before ART and following 6-9 months of ART) were analyzed using paired T-test. Categorical variables were expressed as frequencies (%). The difference in the distribution of variables was checked using Kruskal-Wallis equality-of-populations rank test. Independent variables were analyzed using Mann-Whitney U-test. C hi square test was used to analyze the frequencies of IL-28B genotypes and haplotypes. A p value ≤ 0.05 was used as the criterion for statistical significance. All data generated in the study were analyzed using the MedCalc software.

4. Results

A total of 80 HIV infected individuals from the four south Indian states who were not on antiretroviral therapy were recruited during July 2013 to December 2013 after approval from the Institutional Review Board (IRB Ref Min No 8237 dated 19.03.2013). Seven patients expired during the follow up, while 24 were not on ART lost to follow up. Finally 49 HIV-1 infected individuals who full filled the inclusion criteria who were initiated on ART and followed up for 6-10 months . The mean age of these 49 individuals were 46.21 $\pm$ 10.61. Majority of the subjects were from the state of Tamil Nadu (61.2%) with remaining from Andhra Pradesh and Kerala. The demographic data of the patients recruited in to the study were shown in Table 17.

Table 17: Demographic details of the HIV infected individuals

SL No	State	Total (%)	Male	Female	Age(Mean+SD)
1	Tamil Nadu	30(61.2%)	16	14	43 $\pm$ 10
2	Andhra Pradesh	18(36.7%)	12	6	42 $\pm$ 10
3	Kerala	1(2%)	1	0	51

Controls:

Age and Sex matched HIV sero-negative healthy controls were also recruited. All the 30 healthy individuals were from Tamil Nadu of mean age 42years($\pm$10) were recruited

HIV-1 infected individuals under different antiretroviral therapy regimen

Majority (77%) of the patients were on Zidovidue and Lamivudine with either Nevirapine or Efavirenz. The remaining were on Tenofovir regimen. This data is shown in table 18.

Table 18: Patients under different ART Regimen

Initial ART regimen	Number of cases
Zidovudine + Lamivudine + Efavirenz	10
Zidovudine + Lamivudine + Nevirapine	28
Tenofovir + Lamivudine + Nevirapine	4
Tenofovir + Lamivudine + Efavirenz	4
Tenofovir +Emtricitabine + Efavirenz	1
Tenofovir + Lamivudine + Efavirenz	1
Tenofovir + Emtricitabine + Efavirenz	1

4.1. CD4+ T cell and CD8+ T cell Estimation

4.1.1. Absolute CD4+ and CD8+ T-cell counts, CD4/CD3% and CD4/CD8 ratio before and after 6-9 months of ART.

The absolute CD4+ and CD8+ T-cell counts, CD4/CD3%, CD8/CD3% and CD4/CD8 ratio before and after 6-9 months of ART were analyzed by Paired samples T-test. There was a significant increase ($p = < 0.001$) in the median absolute CD4+ T cell count, CD4/CD3% and there was significant reversal of CD4/CD8 ratio following 6-9 months of ART as shown in the table 19 and figure 12.

Table 19: Cell counts in HIV-1 infected individuals before and after initiation of ART

Parameter	Before ART Median(range)	6-9 months after ART Median (range)	p-value
CD4+ T-cell	147(4-370)	252(17-769)	<0.001
CD4/CD3%	14.3(0.5-4.5)	20.99(4.4-58.8)	<0.001
CD8+ T- cell	775(132-2000)	942(187-2000)	<0.001
CD8/CD3%	78.8(50.3-102.4)	73.54(36.9-89.3)	<0.001
Average CD3	1008(172-2759)	1367(256-3500)	<0.001
CD4/CD8 ratio	0.18(0.01-0.86)	0.28(0.06-1.6)	<0.001

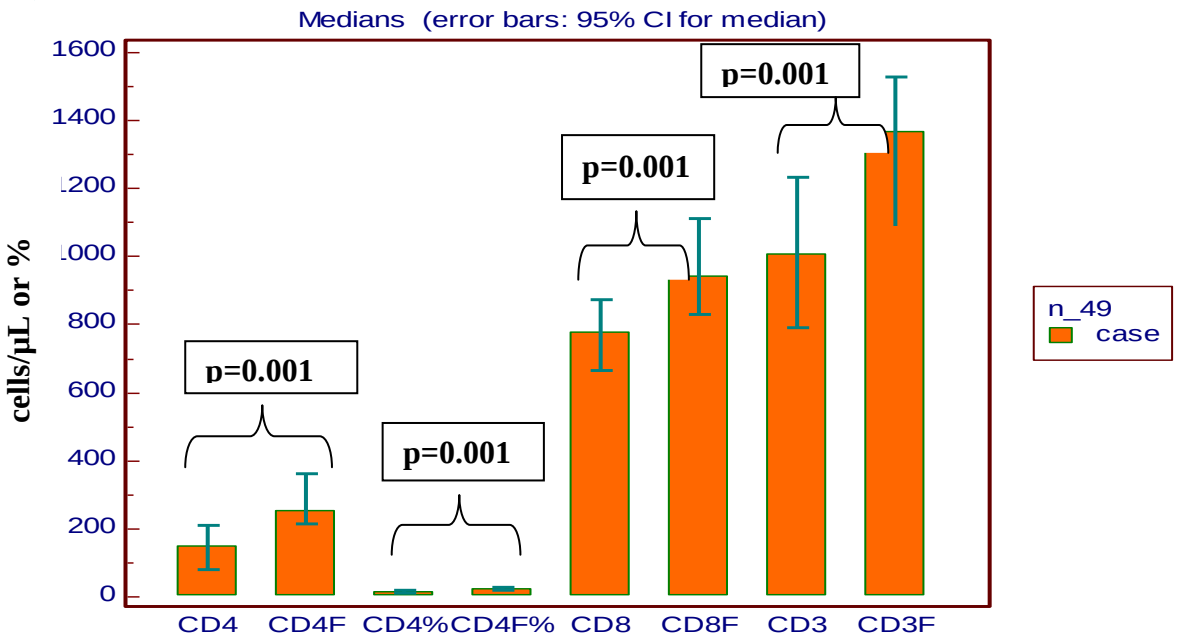


Figure 12: Bar diagram showing the significant increase in CD4+, CD4/CD3%, CD8+ T cell counts and total CD3+ T cell after 6-9 months of ART.

4.2. IL-28B polymorphisms

The IL-28B detection was done by PCR-RFLP as described previously with PBMC samples from all the 49 HIV infected individuals. The gel documentation of PCR amplification product of rs12979860 and rs8099917 SNP is shown in figure 13 and 14 respectively.

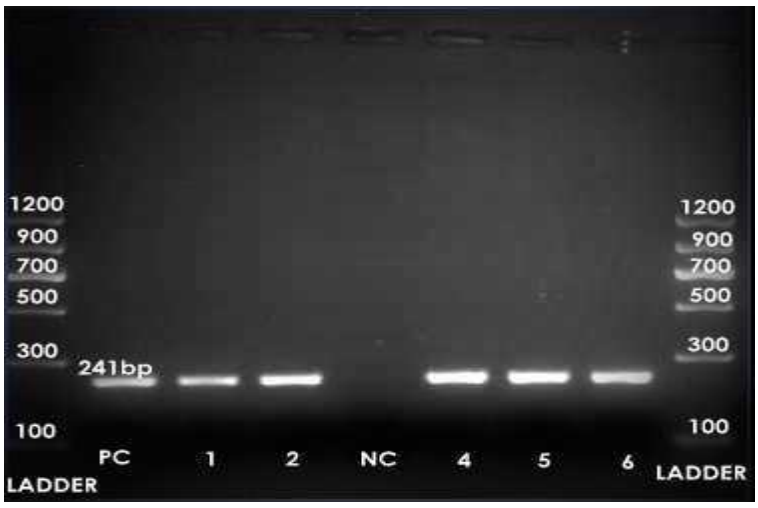


Figure 13: A representative gel picture with rs12979860 PCR amplification product .

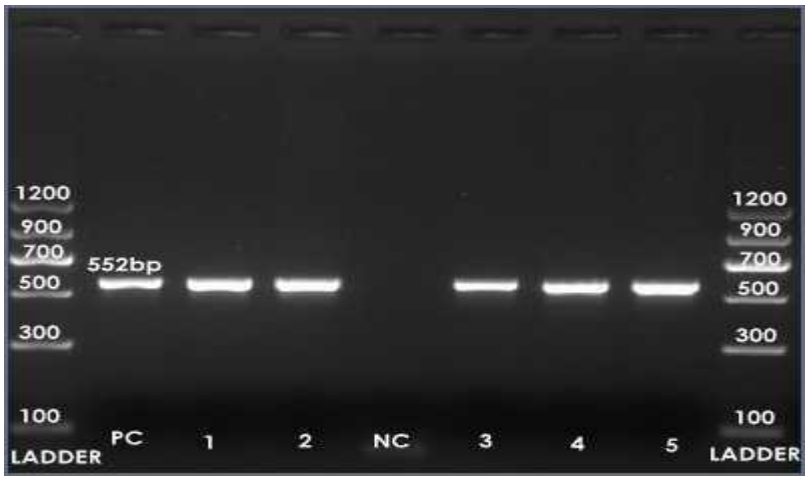
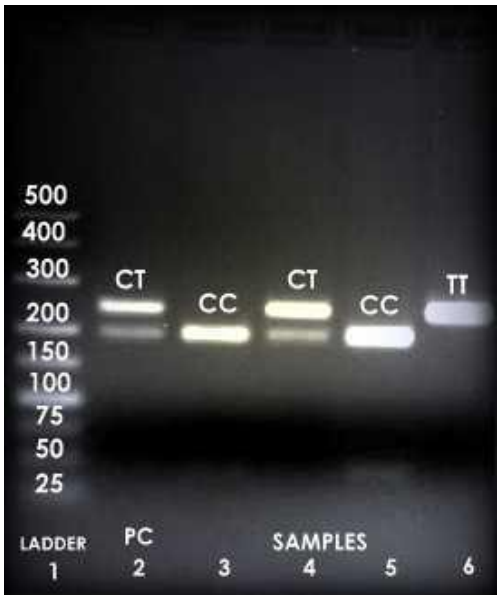


Figure 14: A representative gel picture with rs8099917 PCR amplification product.

4.2.1. RFLP

The amplified products were used for RFLP as described previously(178). The gel documentation picture of rs12979860 and rs8099917 digested products of the amplicons and their different genotypes are shown in figure 15 and 16 respectively**.**



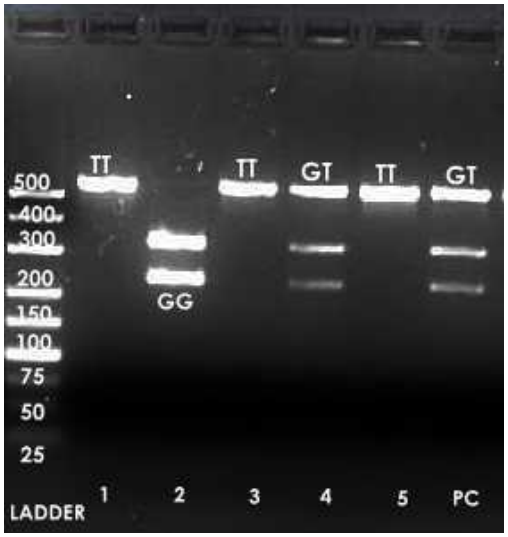
Digested product size

CC- 196bp, 45bp

CT- 241bp, 196bp, 45bp

TT- 241 bp

Figure15: A representative gel picture of RFLP for detection of rs12979860 genotypes.



Digested product size

TT - 552bp,

GT- 552bp, 322bp,230bp

GG- 322 bp, 230bp

Figure 15: A representative gel picture of RFLP for detection of rs8099917 genotypes.

4.3. Distribution of IL-28B genotypes in HIV infected individuals

Among the 49 HIV infected individuals the frequency of CC genotype at rs1297960 was found to be 32 (65%), compared to CT 15(31%) and TT 2(4%). The frequency of wild type CC genotype by Chi-Square test shows a significant (p= <0.001) higher frequency when compared to other SNPs CT and TT. The diagrammatic representation of the distribution of genotypes is shown in figure 16.

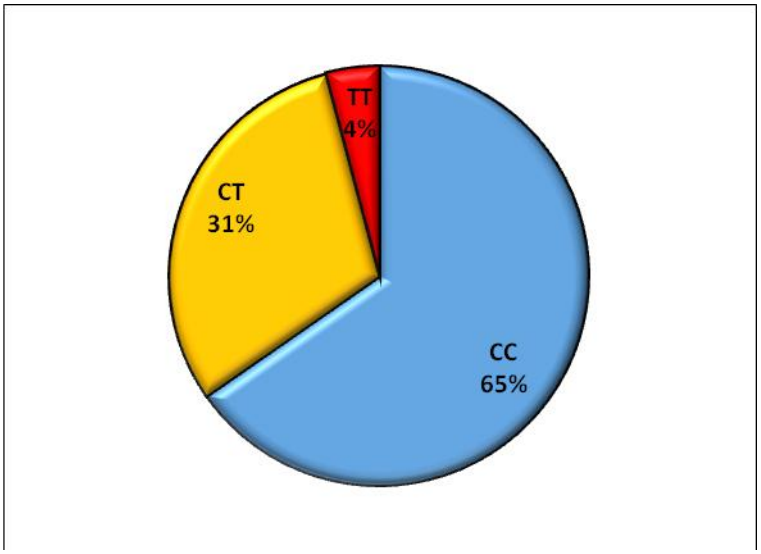


Figure 16: Frequency distribution of rs1297960 polymorphism among HIV-1 infected individuals

Among the 49 HIV infected individuals the frequency of TT genotype at rs8099917 was found to be 39(80%), compared to GT 9(18%) and GG 1(2%). The frequency of wild type TT genotype by Chi-Square test shows a significant (p= <0.0001) higher frequency when compared to other SNPs GT and GG. The diagrammatic representation of the distribution of genotypes is shown in figure 17.

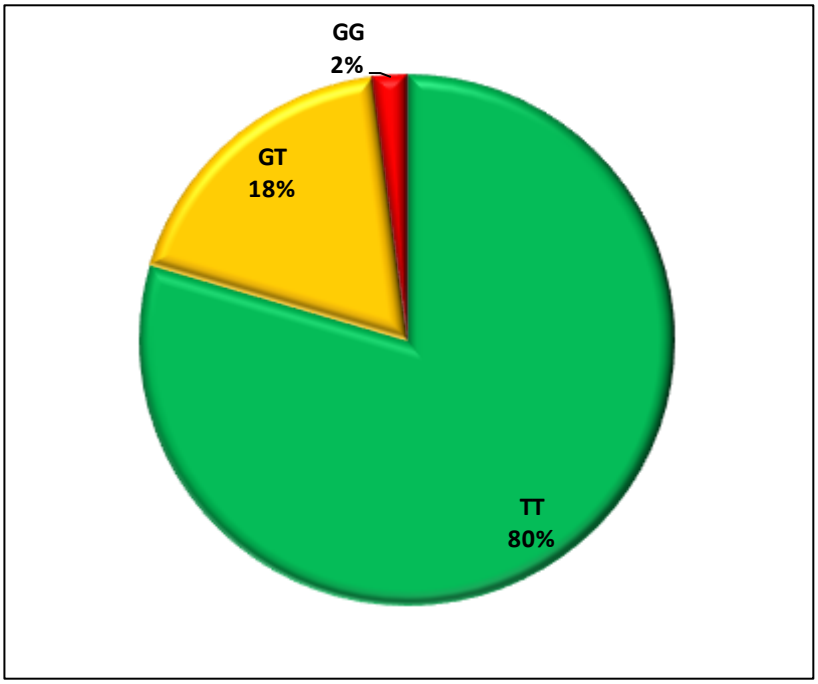


Figure 17: Frequency distribution of rs8099917 polymorphism among HIV-1 infected individuals

4.3.1. Distribution of haplotypes in HIV infected individuals

The frequency of haplotype in HIV infected individuals showed CC/TT 31(64.6%), CT/GT 8(16.7%), CT/TT 7(14.6%), TT/GG 1(2%) and TT/GT 1(2%) as shown in figure 18.The frequency of wild genotype is significantly(p= <0.001) higher compared to other haplotypes as analyzed by Chi-Square test.

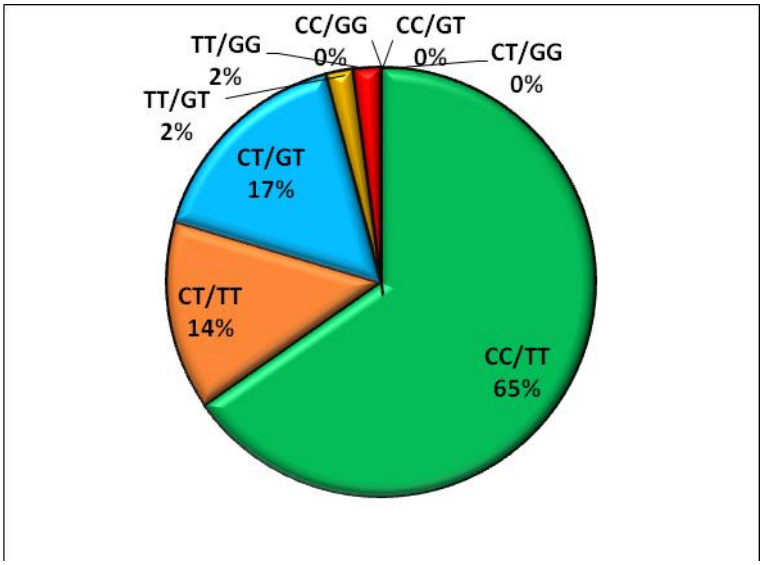


Figure 18: Frequency distribution of rs12979860 and rs8099917 haplotypes in HIV infected individuals

Among the 30 controls the frequency of CC genotype at rs1297960 was found to be 18(60%), compared to CT 10(33%) and TT 2(7%). The frequency of wild type CC genotype by Chi-Square test shows a significant (p= 0.005) higher frequency when compared to other SNPs CT and TT .The diagrammatic representation of the distribution of genotypes is shown in figure 19.

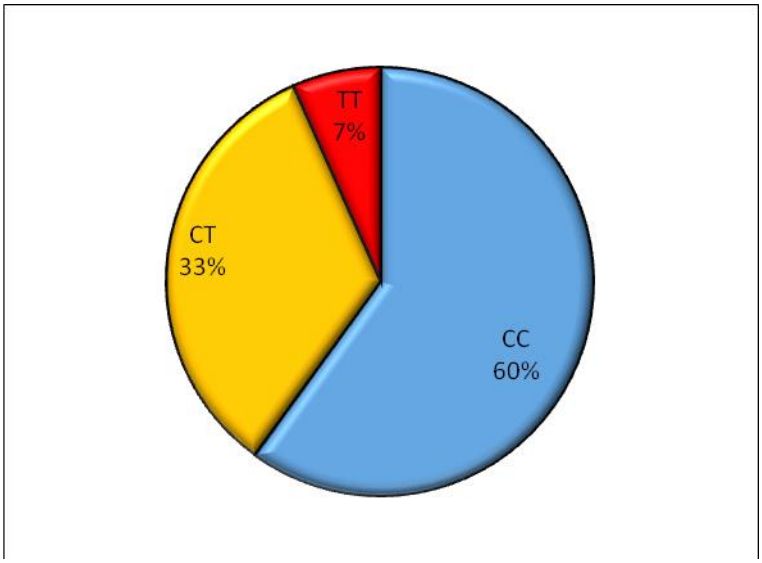


Figure 19: Frequency distribution of rs1297960 polymorphism in HIV seronegative healthy controls

Among the 49 HIV infected individuals the frequency of TT genotype at rs8099917 was found to be 23(77%), compared to GT 7(23%) and GG 0(0%). The frequency of wild type TT genotype by Chi-Square test shows a significant (p= 0.02) higher frequency when compared to other SNPs GT and GG. The diagrammatic representation of the distribution of genotypes is shown in figure 20.

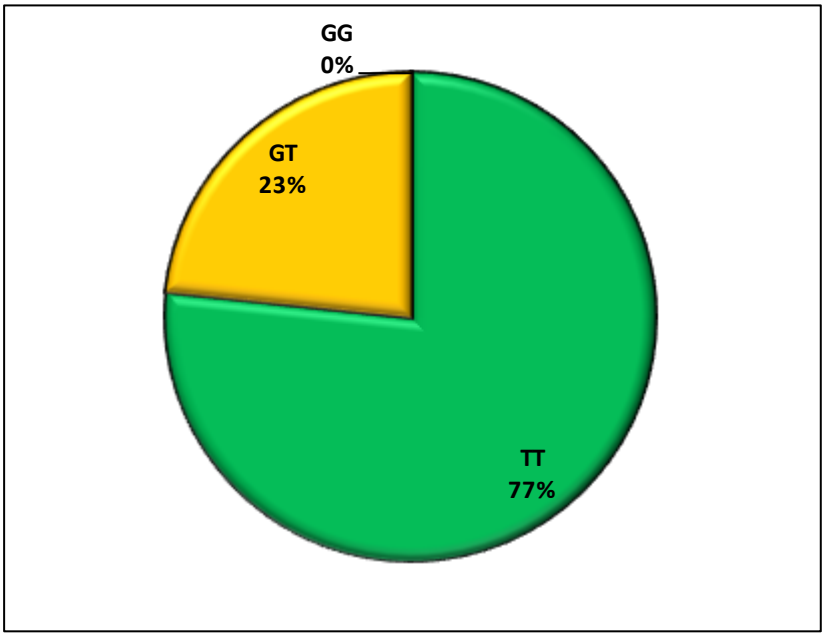


Figure 20: Frequency distribution of rs8099917 polymorphism in HIV seronegative controls

4.3.2. Distribution of haplotype in HIV seronegative controls

The frequency of haplotype in controls showed CC/TT 15(52%), CT/TT 7(24%), CT/GT 3(10%), and TT/GT 1(4%) as shown in figure 21. The frequency of wild genotype is significantly (p= <0.001) higher compared to other haplotypes as analyzed by Chi-Square test.

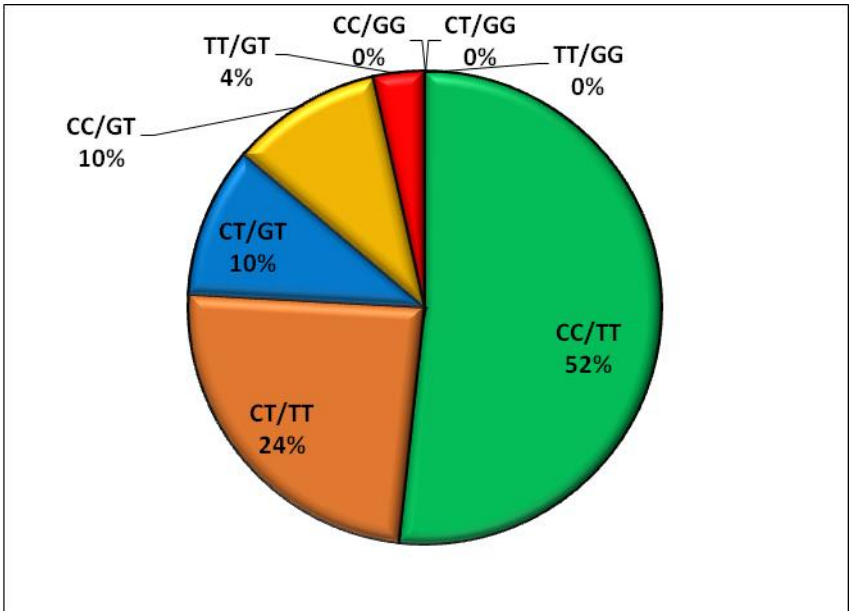


Figure 21: Frequency distribution of rs12979860 and rs8099917 haplotypes in controls

4.4. Association of TT/GG with Immune reconstitution inflammatory syndrome

Out of 49 HIV infected individuals recruited, a 65 years old female from Andhra Pradesh on Zidovudine, Lamivudine and Nevirapine, reported with Immune reconstitution inflammatory syndrome and *Pneumocystis carinii* pneumonia following 1 month of ART. PCR-RFLP revealed homozygous TT genotype of rs12979860 SNP and homozygous GG genotype of rs8099917 SNP. Table 20 summarizes the CD4 counts before ART, on presentation IRIS symptoms and follow up.

Table 20: CD4/CD8 estimation and IL-28B plasma level before ART, during IRIS and Follow-up

	Absolute CD4+(cells/µL)	CD4/CD3%	Absolute CD8+(cells/µL)	CD8/CD3%	CD4/CD8 ratio	IL-28B Plasma (pg/mL)
Before ART	48	4.92	874	89.55	0.05	137.7
IRIS	43	12.32	290	83.09	0.15	16.15
Follow-up	107	9.58	975	87.29	0.11	0
Follow-up2	186	8.51	1996	91.31	0.09	3.80

4.5. The frequency distribution of the IL-28B genotypes in HIV infected individuals and controls

The frequency distribution of the IL-28B genotypes in HIV infected individuals and controls are summarized in the table 21.

Table 21: Frequency and percentage of IL28B polymorphisms in cases and controls

IL-28B polymorphism	Control (n=30) n (%)	p-value	HIV infected individuals (n=49) n(%)	p-value
rs12979860				
• **CC**	18(60)	<0.001	32(65)	<0.001
• **CT**	10(33)		15(31)	
• **TT**	2(7)		2(4)	
rs8099917				
• **TT**	23(77)	<0.001	39(80)	<0.001
• **GT**	7(23)		19(18)	
• **GG**	0(0)		1(2)	

4.6.1. Association of rs12979860 and rs8099917 with CD4 T-cell counts before and after treatment

The association of rs12979860 and rs8099917 and the median absolute CD4+ T-cell counts were analyzed using Kruskal-Wallis test. It was found that these genotypes are not significantly associated with absolute CD4+ T-cell counts before and after ART. The interpretation is summarized in the table 22.

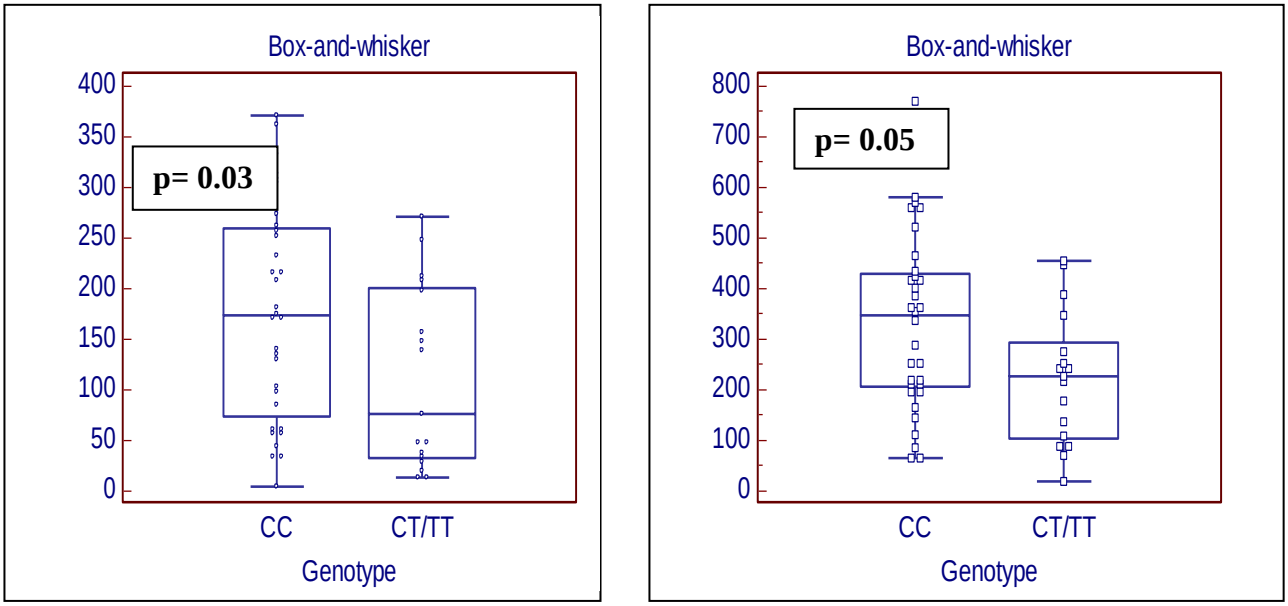
Table 22: Association of rs12979860 and rs8099917 genotypes with CD4 T-cell counts before and after treatment

IL-28B polymorphism	Cases (n=49) n(%)	Pre Rx CD4 count Median (range)	p-value (Pre ART)	Post Rx CD4 Count Median(range)	p-value (Post ART)
rs12979860					
• **CC**	32(65)	173.5(4.0-37)	0.8	345(64-769)	0.1
• **CT**	15(31)	138.0(13-27)		242(17-453)	
• **TT**	2(4)	62(48-76)		166(107-225)	
rs8099917					
• **TT**	39(80)	156.0(4-37)	0.4	275(64-769)	0.5
• **GT**	9(18)	111.50(13-24)		242(17-453)	
• **GG**	1(2)	122.50(48-19)		107	

4.6.2. Association of genotypes with absolute CD4+ T-cell count

The association of wild type genotypes CC and CT or TT at rs12979860 and TT and GT or GG at rs8099917 with the median absolute CD4 counts before and after 6-9 months of ART was analyzed by using Kruskal- Wallis test. The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. The horizontal line extends from the minimum to the maximum value, excluding the outliers

which are displayed as separate points. The wild genotype CC at rs12979860 showed a significant (p-value = 0.03) higher CD4+ T cell count when compared to other genotypes CT and TT. The absolute CD4+ T-cell counts in comparison to genotypes are represented by box and whisker in Figure 22.



(a) **(b)**

Figure 22: Box and whisker showing the association of CC genotype CD4+ T-cell counts before(a) and after(b) 6-9 months of ART.

4.6.3. Association of haplotypes with absolute CD4+ T-cell count.

The haplotypic association of wild type haplotypes CC/TT and CT/GT, CT/TT, TT/GT and TT/GG at rs12979860 and rs8099917 with median absolute CD4 counts before and after 6-9 months of ART was analyzed by using Kruskal- Wallis test. The wild haplotype CC/TT showed a significant (p-value= 0.03) higher CD4+ T cell count when compared to other haplotypes.

The absolute CD4+ T-cell counts before and after 6-9 months of ART in comparison to haplotypes are represented by box whisker in Figure 23.

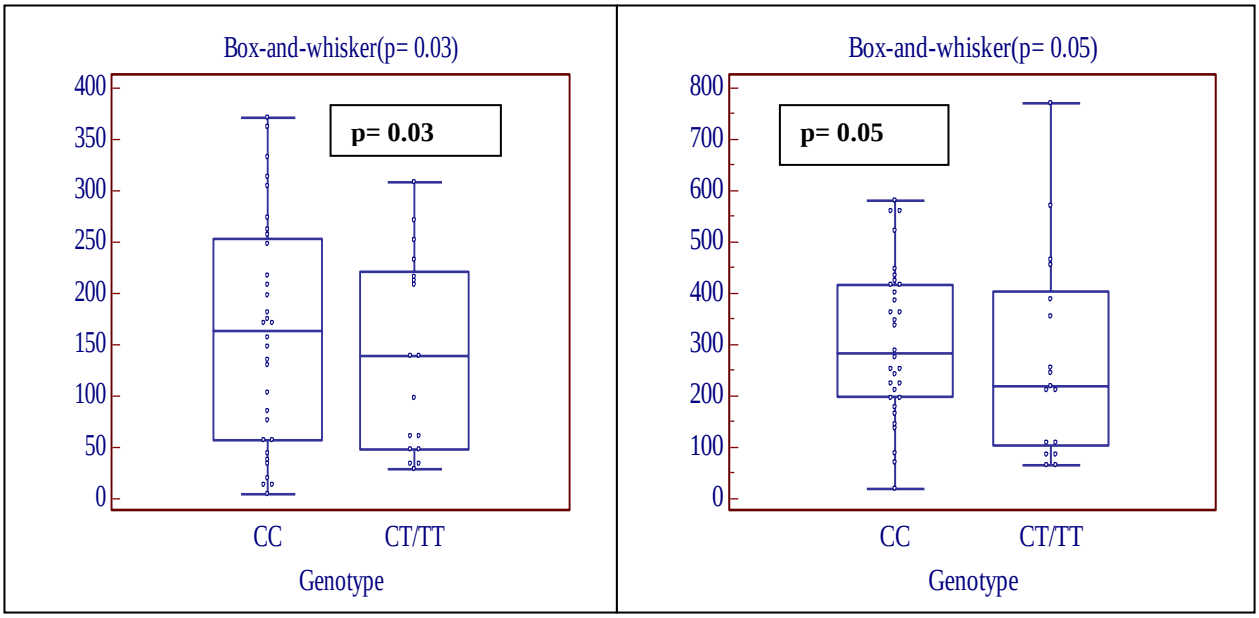


Figure 23: Box and whisker showing the association of CC/TT haplotype CD4+ T-cell counts before and after 6-9 months of ART.

4.6.4. Association of rs12979860 and rs8099917 SNPs with CD4/CD3% T-cell counts before and after treatment.

The association of rs12979860 and rs8099917 SNPs and the median CD4/CD3% counts were analyzed using Kruskal-Wallis test. It was found that these genotypes are not significantly (p= >0.05) associated with absolute CD4CD3%+ T-cell counts before and after ART. The interpretation is summarized in the table 23.

Table 23: Association of rs12979860 and rs8099917 SNPs with CD4/CD3% counts before and after treatment.

IL-28B polymorphism	Cases (n=49) n(%)	Pre ART CD4/CD3% count Median (range)	p-value (Pre ART)	Post Rx CD4/CD3% Count Median(range)	p-value (Post ART)
rs12979860					
• **CC**	32(65)	16.11(0.52-26.8)	0.2	24.47(7.03-58.81)	0.06
• **CT**	15(31)	12.81(2.41-43.48)		15.84(4.44-41.06)	
• **TT**	2(4)	10.19(4.91-15.47)		22.76(9.58-35.94)	
rs8099917					
• **TT**	39(80)	15.1(0.52- 26.8)	0.5	21.11(6.27-58.81)	0.3
• **GT**	9(18)	14.32(4.37-43.48)		17.25(4.44-35.94)	
• **GG**	1(2)	4.91		9.58	

4.6.5. Association of genotypes with CD4/CD3% counts before and after treatment.

The association of wild type genotypes CC and CT or TT at rs12979860 and TT and GT or GG at rs8099917 with median CD4/CD3% before and after 6-9 months of ART was analyzed by using Kruskal- Wallis test. The wild genotype CC at rs12979860 showed a significant (p-value= 0.03) higher CD4/CD3% when compared to other genotypes CT and TT.

The association of genotypes with CD4/CD3% before and after 6-9 months of ART in are represented by box & whisker in Figure 24.

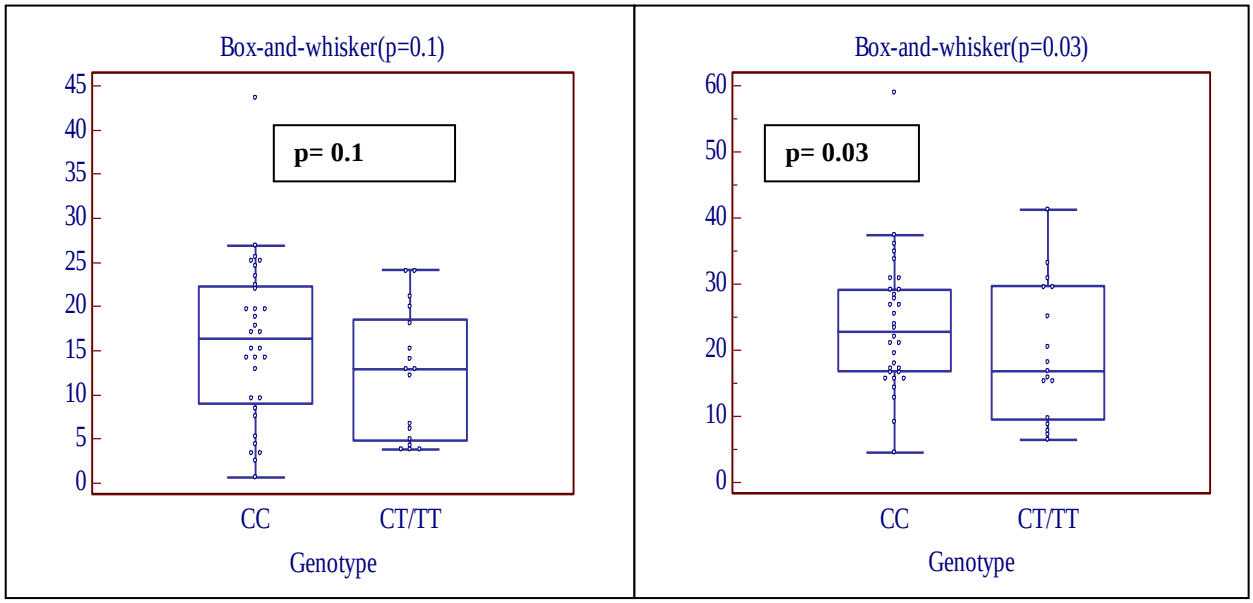


Figure 24: Box and whisker plot to showing the association of rs12979860 genotypes with CD4/CD3% counts before and after treatment.

4.6.6. Association of rs12979860 and rs8099917 with CD8 T-cell counts before and after treatment

The association of rs12979860 and rs8099917 and the median absolute CD4+ T-cell counts were analyzed using Kruskal-Wallis test. It was found that these genotypes are not significantly associated with absolute CD8+ T-cell counts before and after ART. The interpretation is summarized in the table 24.

Table 24: Association of rs12979860 and rs8099917 with CD8 T-cell counts before and after treatment

IL-28B polymorphism	Cases (n=49) n(%)	Pre Rx CD8 count Median (range)	p-value (Pre ART)	Post Rx CD8 Count Median(range)	p-value (Post ART
rs12979860					
• **CC**	32(65)	796(239-2000)	0.3	928(187-2000)	0.1
• **CT**	15(31)	674(132-1582)		1145(272-2000)	
• **TT**	2(4)	600.5(327-874		600.5(327-874)	
rs8099917					
• **TT**	39(80)	750(73.95-2000)	0.4	942(187-2000)	0.4
• **GT**	9(18)	698.5(132-1312)		861(298-2000)	
• **GG**	1(2)	874		975	

4.6.7. Association of haplotypes with CD8+ T-cell count before and after 6-9 months of ART

The haplotypic association of wild type haplotype CC/TT and the other haplotypes CT/GT, CT/TT, TT/GT and TT/GG at rs12979860 and rs8099917 with median absolute CD8+ T-cell counts before and after 6-9 months of ART by using Kruskal- Wallis test. No significant(p= >0.05) association was found out between haplotypes and absolute CD8+ T-cell count.

The association of haplotypes with absolute CD8+ T-cell counts before and after 6-9 months of ART are represented by box-whisker plot in Figure 25.

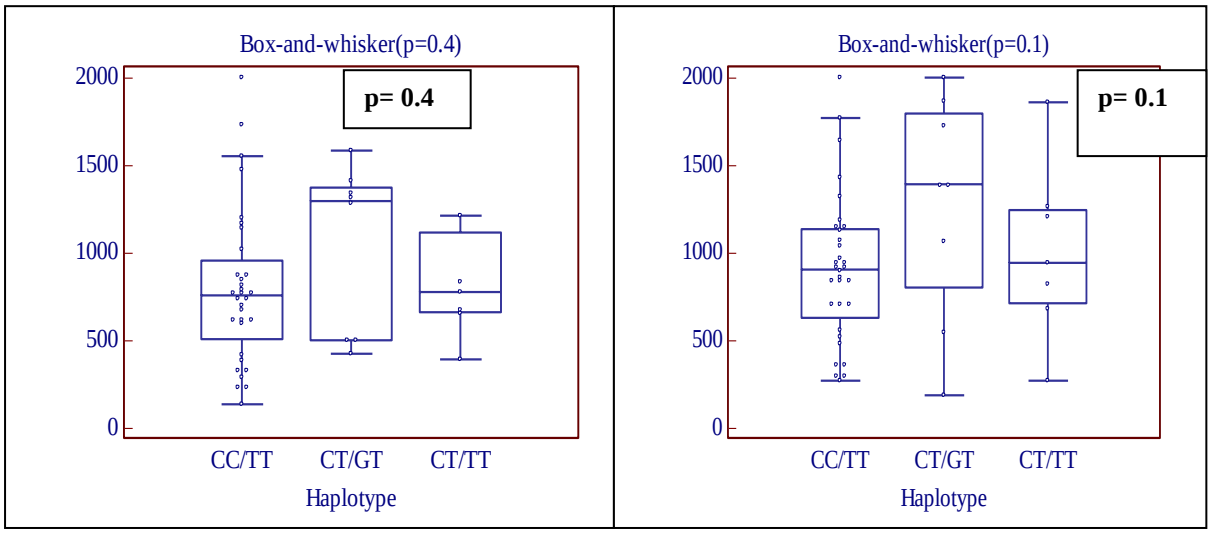


Figure 25: Box and whisker plot showing the association of haplotypes with absolute CD8+ T-cell count before and after 6-9 months of ART.

4.6.8. Association of rs12979860 and rs8099917 with CD8/CD3% counts before and after treatment

The association of rs12979860 and rs8099917 and the absolute CD8/CD3% counts were analyzed using Kruskal-Wallis test. It was found that these genotypes are not significantly associated with absolute CD8/CD3% counts before and after ART. The interpretation is summarized in the table 25 below.

Table 25: Association of rs12979860 and rs8099917 SNPs with CD8/CD3% T-cell before and after treatment.

IL-28B polymorphism	Cases (n=49) n(%)	Pre Rx CD8/CD3% count Median (range)	p-value (Pre ART)	Post Rx CD8/CD3% Count Median(range)	p-value (Post ART
rs12979860					
• **CC**	32(65)	77.62(57-102.41)		72.6(36.86-88.5)	
• **CT**	15(31)	81.84(50.33-96.1)	0.5	75.05(51.71-89.3)	0.5
• **TT**	2(4)	78.07(66.59-89.54)		72.24(57.19-87.29)	
rs8099917					
• **TT**	39(80)	78.66(57-102.41)		73.54(36.86-89.3)	
• **GT**	9(18)	79.32(50.33-84.53)	0.4	72.79(57.14-79)	0.2
• **GG**	1(2)	89.54		87.29	

4.6.9 Association of rs12979860 and rs8099917 SNPs with CD4/CD8 ratio before and after treatment.

The association of rs12979860 and rs8099917 SNPs and median CD4/CD8 ratio were analyzed using Kruskal-Wallis test. It was found that these genotypes are not significantly associated with median CD4/CD8 ratio before and after ART. The interpretation is summarized in the table 26.

Table 26: Association of rs12979860 and rs8099917 SNPs with CD4/CD8 ratio before and after treatment.

IL-28B polymorphism	Cases (n=49) n(%)	Pre Rx CD4/CD8 count Median (range)	p-value (Pre ART)	Post Rx CD4/CD8 Count Median(range)	p-value (Post ART
rs12979860					
• **CC**	32(65)	0.19(0.01-0.38)		0.32(0.06-1.6)	
• **CT**	15(31)	0.16(0.03-0.86)	0.5	0.22(0.06-0.79)	0.3
• **TT**	2(4)	0.14(0.05-0.23)		0.37(0.11-0.63)	
rs8099917					
• **TT**	39(80)	0.17(0.01-0.38)		0.28(0.06-1.6)	0.4
• **GT**	9(18)	0.23(0.06-0.63)	0.1	0.24(0.06-0.63)	
• **GG**	1(2)	0.05		0.11	

4.6.10. Association of haplotypes with CD4/CD8 before and after 6-9 months of ART.

The haplotypic association of wild type haplotypes CC/TT and CT/GT, CT/TT, TT/GT and TT/GG at rs12979860 and rs8099917 SNP with median CD4/CD8 ratio before and after 6-9 months of ART was analyzed by using Kruskal- Wallis test. No significant (p= >0.05) association was found out between haplotypes and CD4/CD8 ratio. The association of absolute CD8+ T-cell counts before and after 6-9 months of ART in with the haplotypes are represented in a Box and whisker plot in Figure 26.

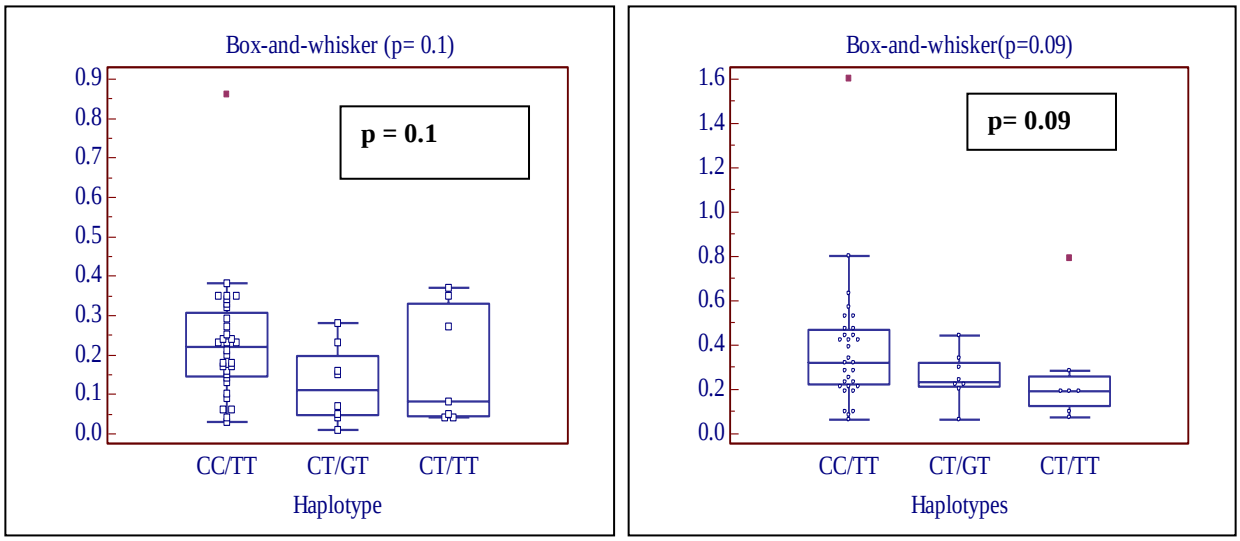


Figure 26: Box and whisker showing the association of haplotype with the CD4/CD8 ratio before and after initiation of ART.

4.7. IL-28B plasma level estimation

IL-28 plasma level was estimated by a Human IL-28B in-house quantitative ELISA kit (R&D Systems, Minneapolis, MN, USA) [Catalog Number: DY5259] as per manufacturer's instructions as described in the methods.

Calculation of concentration

The average of concentration for each sample was calculated. Using standards in 7 dilutions and zero standard, a 4-Parameter logistic(4-PL) curve was generated with concentrations on X-axis and Delta OD values on Y-axis using Gen5 software as shown in figure 27 and the curve fit results are shown in table 27.

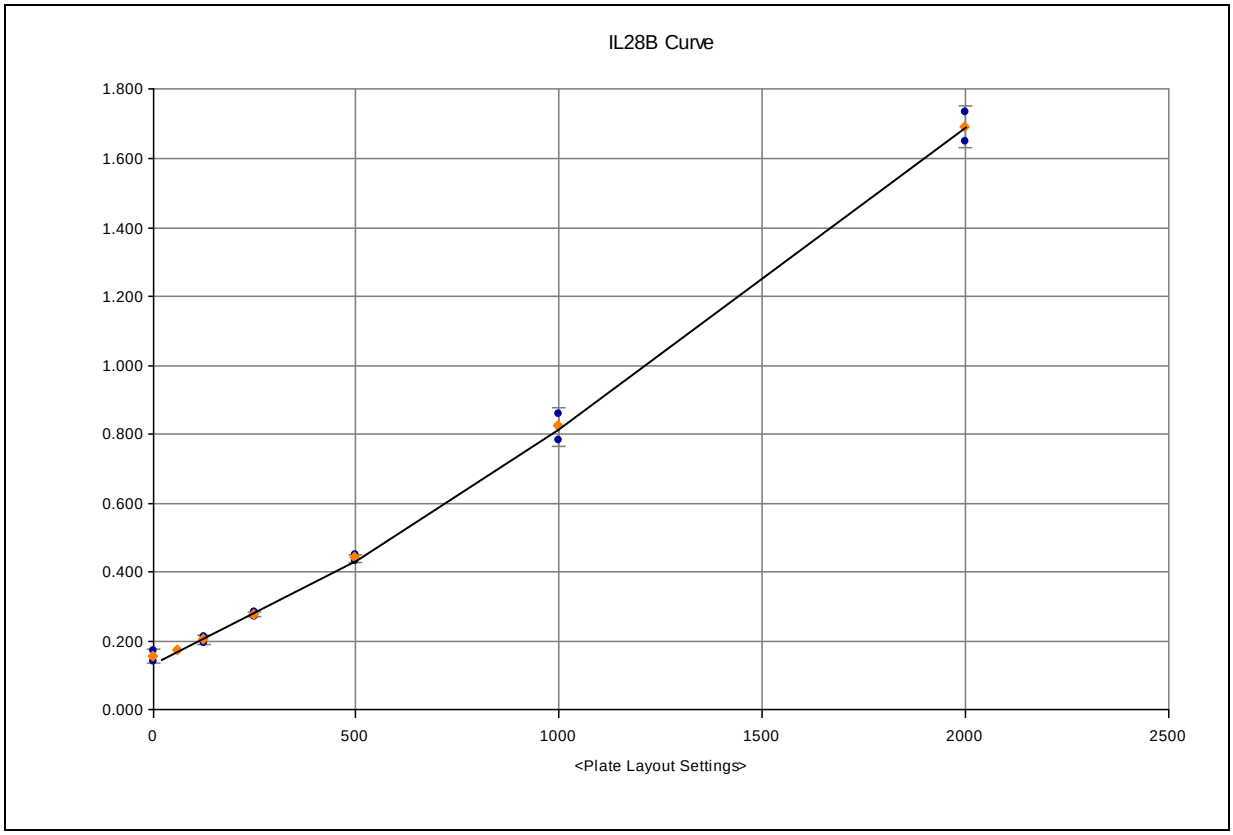


Figure 27: A representative picture of 4-Parameter Logistic (4-PL) Curve constructed for standards with 7 dilutions using Gen5 software performed in this study.

Table 27: A representative curve fit results calculated by Gen5 software.

Curve Name	Curve Formula	A	B	C	D	R2
IL28B Curve	Y = (A-D)/(1+(X/C)^B) + D	0.155	1.24	2.29E+04	33.5	1

Validation of ELISA:

The inter-assay variation was calculated based on readings of 5 days. The Co-efficient of variation (CV%) calculated for standards of concentrations 500pg/ml, 125pg/ml and 62.5pg/ml were 2.3%, 12.7% and 37.5% respectively.

4.7.1. Association of rs12979860 and rs8099917 SNPs with IL-28B plasma level before and after 6-9 months treatment in HIV infected individuals.

The association of rs12979860 and rs8099917 SNPs with median IL-28B plasma level were analyzed using Kruskal-Wallis test. It was found that these genotypes were not significantly (p = >0.05) associated with median IL-28B plasma level before and after ART. The interpretation is summarized in the table 28.

Table 28: Association of rs12979860 and rs8099917 genotypes with IL-28B plasma level before and after treatment

IL-28B polymorphism	Cases (n=49) n(%)	Pre ART IL-28B plasma level Median (range)	p-value Pre ART	Post ART IL-28B plasma Level Median(range)	p-value Post ART
rs12979860					
• **CC**	32(65)	68.75(0.0-221.26)		69.82(80-490.25)	
• **CT**	15(31)	91.54(0-524.48)	0.5	30.85(0-394.3)	0.1
• **TT**	2(4)	75.19(12.65-137.74)		0(0)	
rs8099917					
• **TT**	39(80)	59.06(0.0-514.48)		61.83(0-490.25)	
• **GT**	9(18)	129.5(912.65-236.46)	0.3	30.850(0-207.13)	0.2
• **GG**	1(2)	137.73		0	

4.7.2. Association of rs12979860 genotypes with IL-28B plasma level in HIV infected individuals.

The association of rs12979860 genotypes with median IL-28B plasma level was analyzed using Kruskal-Wallis test. It was found that these genotypes are not significantly (p = >0.05) associated with median IL-28B plasma level before and after ART. Box and whisker showing the association of rs12979860 with IL-28B plasma level is shown in figure 28.

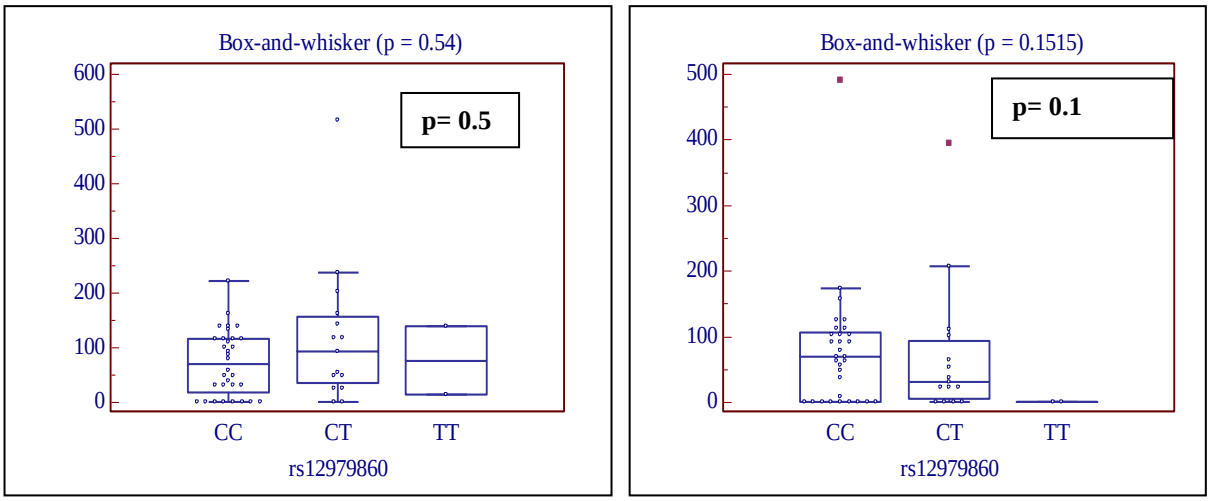


Figure 28: Box and whisker plot to look at the association of genotypes of rs12979860 with IL-28B plasma level in HIV infected individuals.

4.7.3.Association of rs8099917 with IL-28B plasma level in HIV infected individuals

The association of rs8099917 genotypes with median IL-28B plasma level was analyzed using Kruskal-Wallis test. It was found that these genotypes are not significantly (p = >0.05) associated with IL-28B plasma level before and after ART. Box and whisker showing the association of rs809917 with IL-28B plasma level is shown in figure 29.

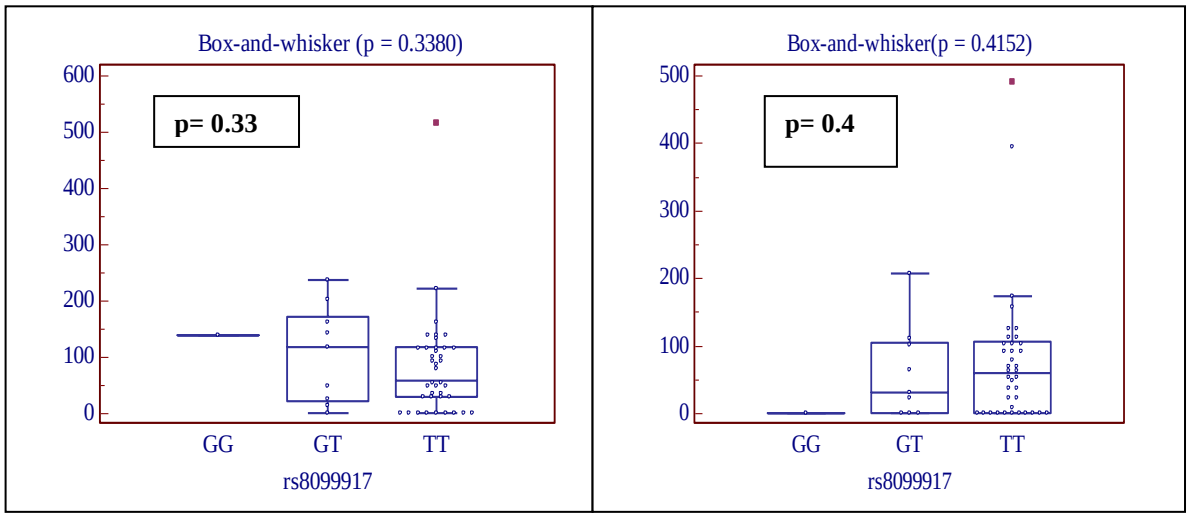


Figure 29: Box and whisker plot to look at the association of genotypes of rs8099917 with IL-28B plasma level in HIV infected individuals.

4.7.4. Association of haplotype CC/TT and other haplotypes with IL-28B plasma level in HIV infected individuals before ART.

The association of haplotypes with median IL-28B plasma level was analyzed using Kruskal-Wallis test. It was found that they are not significantly (p = >0.05) associated with median IL-28B plasma level before ART. Box and whisker showing the association of haplotypes with IL-28B plasma level is shown in figure 30.

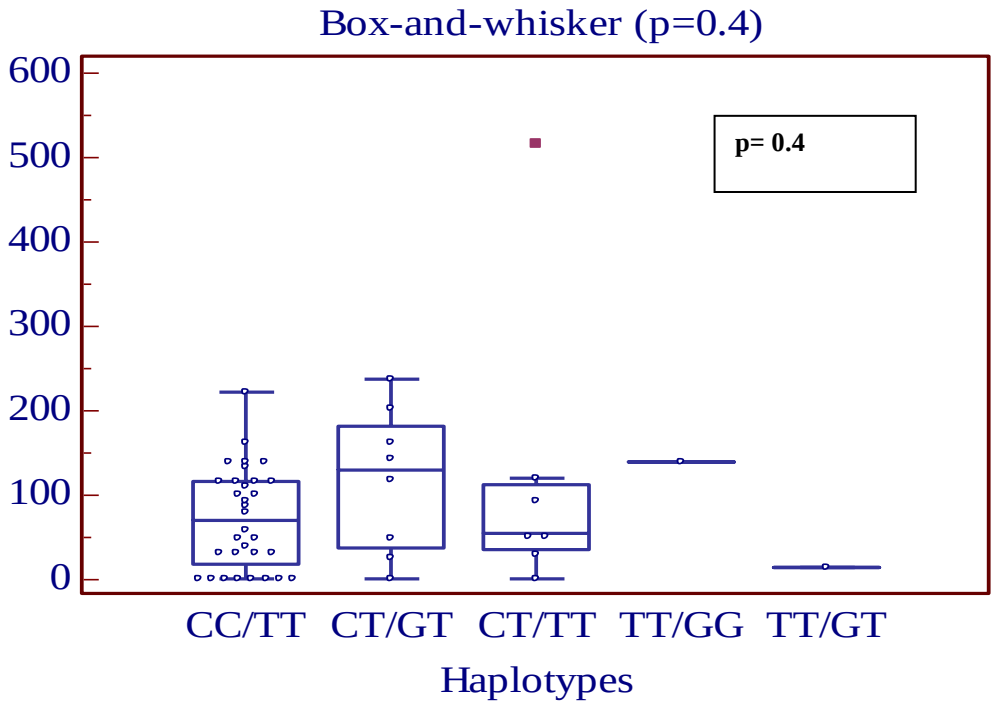


Figure 30: Box and whisker showing the association of haplotype CC/TT and other haplotypes with IL-28B plasma level in HIV infected individuals.

4.7.5. Association of haplotype CC/TT and other haplotypes with IL-28B plasma level in HIV infected individuals after 6-9 months ART.

The association of haplotypes with median IL-28B plasma level was analyzed using Kruskal-Wallis test. It was found that they are not significantly (p = >0.05) associated with median

IL-28B plasma level after ART. Box and whisker showing the association of haplotypes with IL-28B plasma level is shown in figure 31.

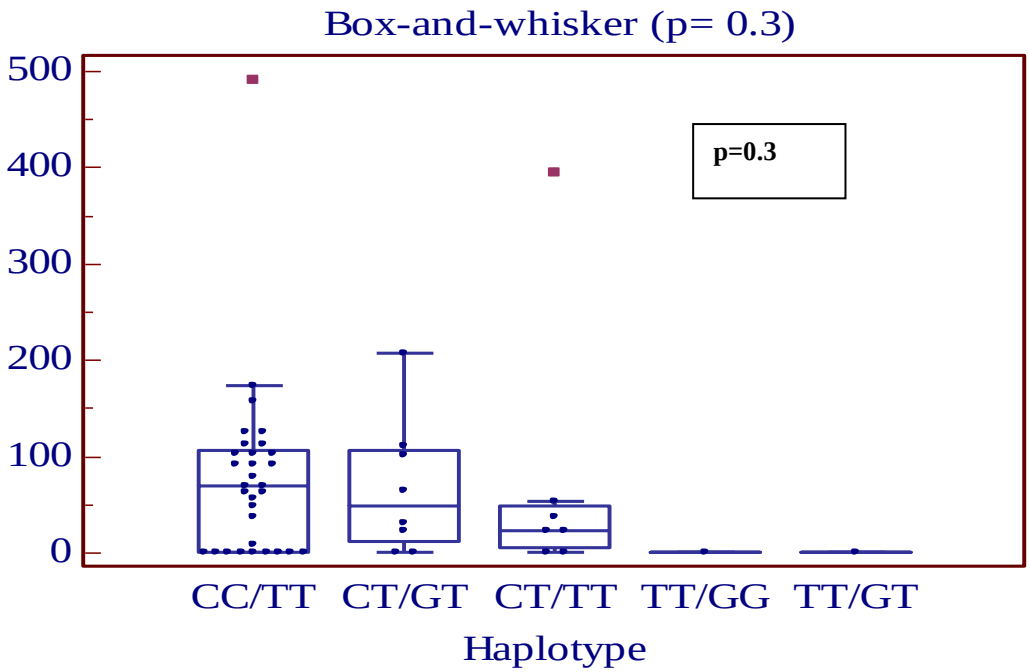


Figure 31: Box and whisker showing the association of haplotype CC/TT and other haplotypes with IL-28B plasma level in HIV infected individuals.

4.7.5.Association of rs12979860 SNP with IL-28B plasma level in controls.

The association of rs12979860 genotypes with median IL-28B plasma level in controls was analyzed using Kruskal-Wallis test. It was found that these genotypes are not significantly (p = >0.05) associated with median IL-28B plasma level before and after ART. Box and whisker showing the association of rs12979860 with IL-28B plasma level in controls is shown in figure 32.

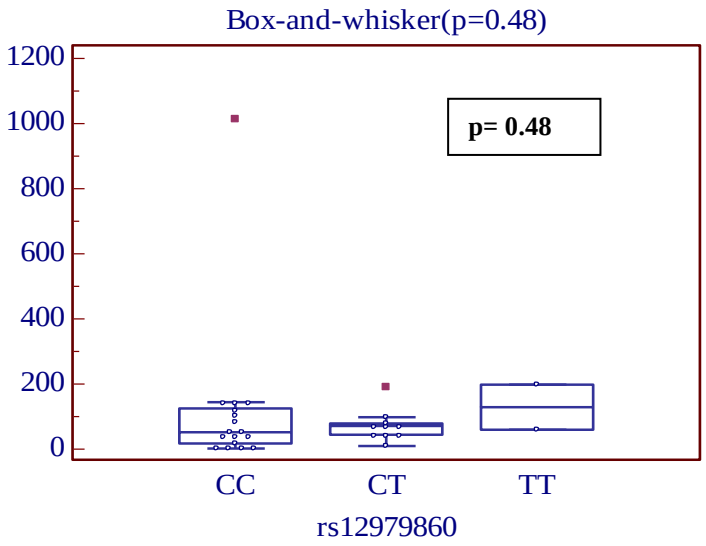


Figure 32: Box-whisker plot comparing the IL-28B plasma levels with the genotypes at rs12979860 in controls.

4.7.6. Association of rs8099917 genotypes with IL-28B plasma level in controls

The association of rs8099917 genotypes with median IL-28B plasma level in controls was analyzed using Kruskal-Wallis test. It was found that these genotypes are not significantly (p = >0.05) associated with median IL-28B plasma level before and after ART. Box and whisker plot showing the association of rs8099917 with IL-28B plasma level in controls is shown in figure 33.

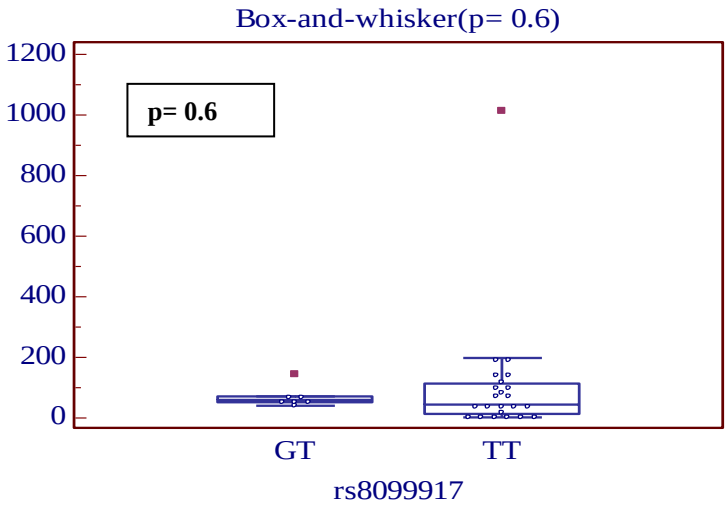


Figure 33: Box-whisker plot comparing the IL-28B plasma levels with the genotypes at rs8099917 in controls.

4.7.7. Association of haplotypes with IL-28B plasma level in controls

The association of haplotypes with median IL-28B plasma level in controls was analyzed using Kruskal-Wallis test. It was found that these genotypes are not significantly (p = >0.05) associated with median IL-28B plasma level before and after ART. Box and whisker showing the association of haplotype with IL-28B plasma level in controls is shown in figure 34.

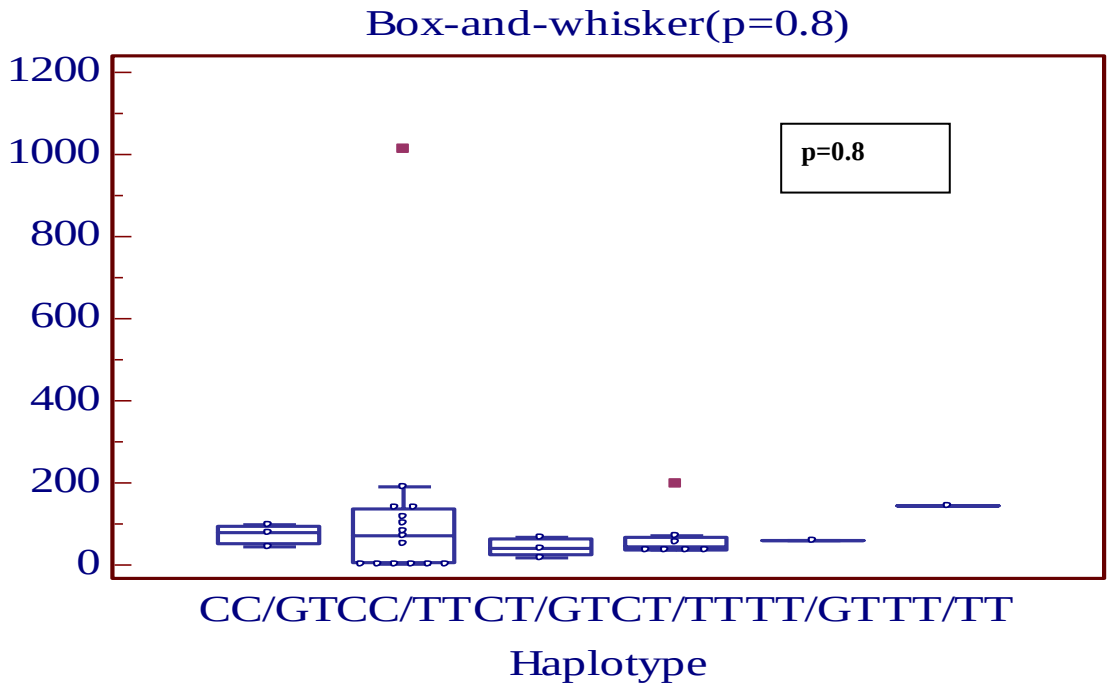


Figure 34: Box-whisker plot comparing the IL-28B plasma levels with the haplotypes in HIV seronegative controls.

4.7.8. IL28B plasma level comparison between cases and control

The association of median IL-28B plasma level in HIV infected individuals and controls was analyzed using Mann-Whitney U test. There is no significant (p = >0.05) difference in IL-28B plasma level HIV infected individuals and controls. Box and whisker showing the difference of IL-28B plasma level in HIV infected individuals and controls is shown in figure 35.

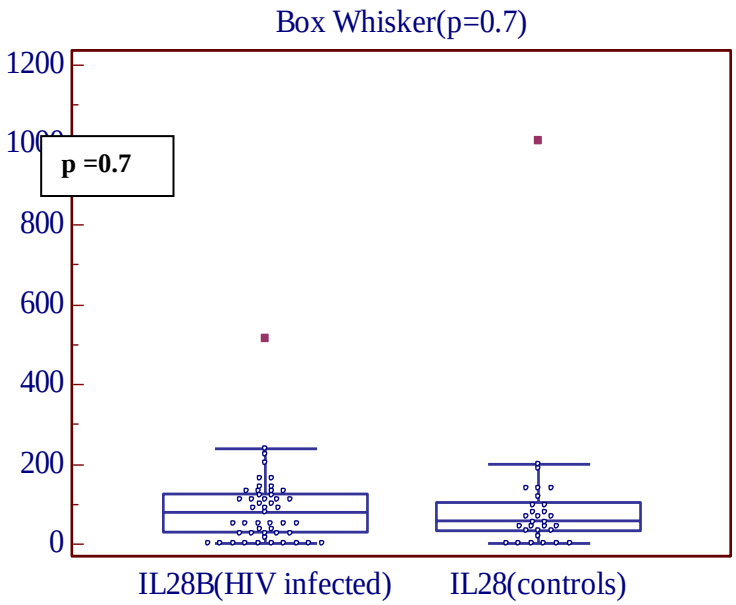


Figure 35: Box-whisker plot comparing the IL-28B plasma levels in cases and controls

4.7.9.Association of haplotypes with IL-28B plasma level of HIV infected individuals before ART

The association of haplotypes with median IL-28B plasma level in HIV infected individuals was analyzed using Kruskal-Wallis test. It was found that these haplotypes are not significantly (p = >0.05) associated with IL-28B plasma level before ART. Box and whisker showing the association of haplotype with IL-28B plasma level in HIV infected individuals before ART is shown in figure 36.

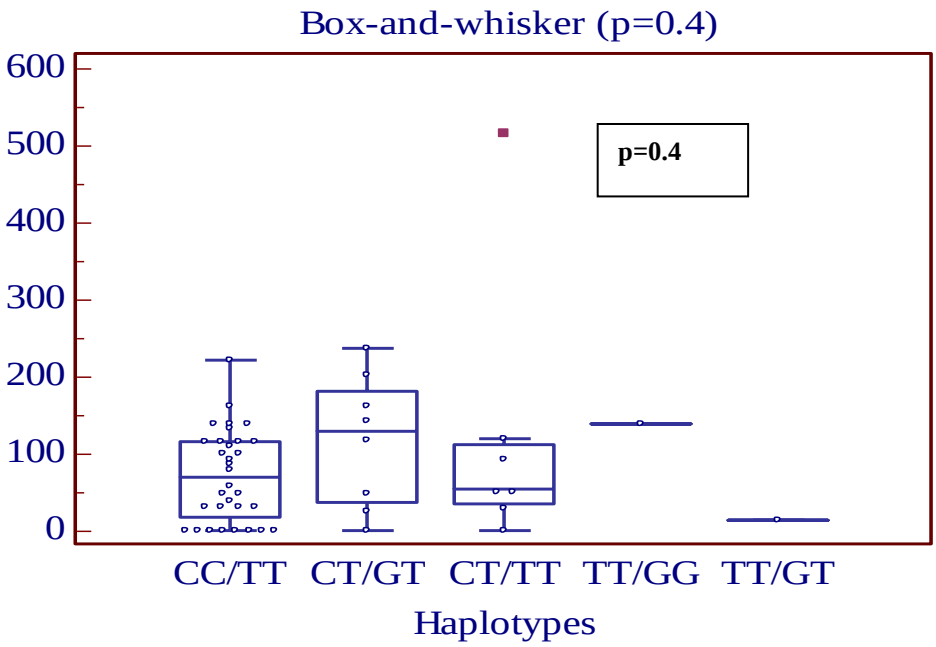


Figure 36: Box-whisker plot comparing the IL-28B plasma levels with the haplotypes in HIV infected individuals.

4.7.10Association of haplotypes with IL-28B plasma level after 6-10 months of ART in HIV infected individuals.

The association of haplotypes with median IL-28B plasma level in HIV infected individuals was analyzed using Kruskal-Wallis test. It was found that these haplotypes are not significantly ($p = >0.05$) associated with IL-28B plasma level after 6-10 months of ART. Box and whisker showing the association of haplotypes with IL-28B plasma level in HIV infected individuals after ART is shown in figure 37.

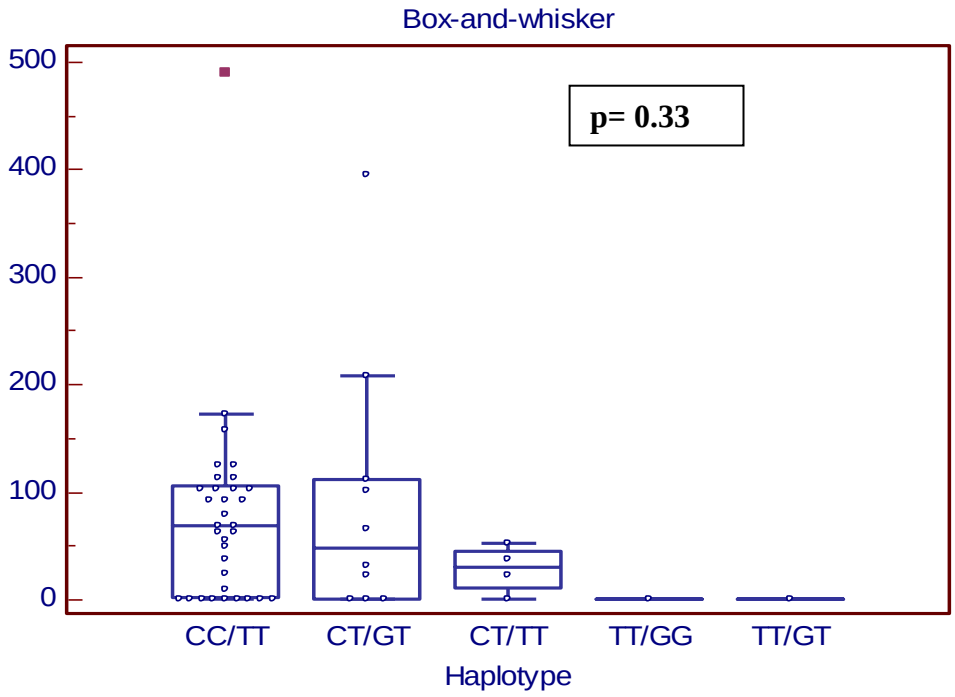


Figure 37: Box-whisker plot comparing the IL-28B plasma levels after 6-9 months of ART with the haplotypes in cases.

4.8.1.Association of IL-28B plasma level with CD4 counts

The association of IL-28B plasma level and absolute CD4+ T-cell counts were analyzed using Spearman's correlation test. R (rho value = 0.6-0.8) is significant and indicates positive correlation. It was found that absolute CD4+ T-cell count is not significantly (p = >0.05 & R= -0.142)) associated with median IL-28B plasma level before and after ART(p= >0.05, R= 0.256). IL-28B plasma level does not have any effect on absolute CD4+ T-cell count before ART and increase in absolute CD4+ T-cell count after 6-9 months of ART. The interpretation is summarized in the figure 38.

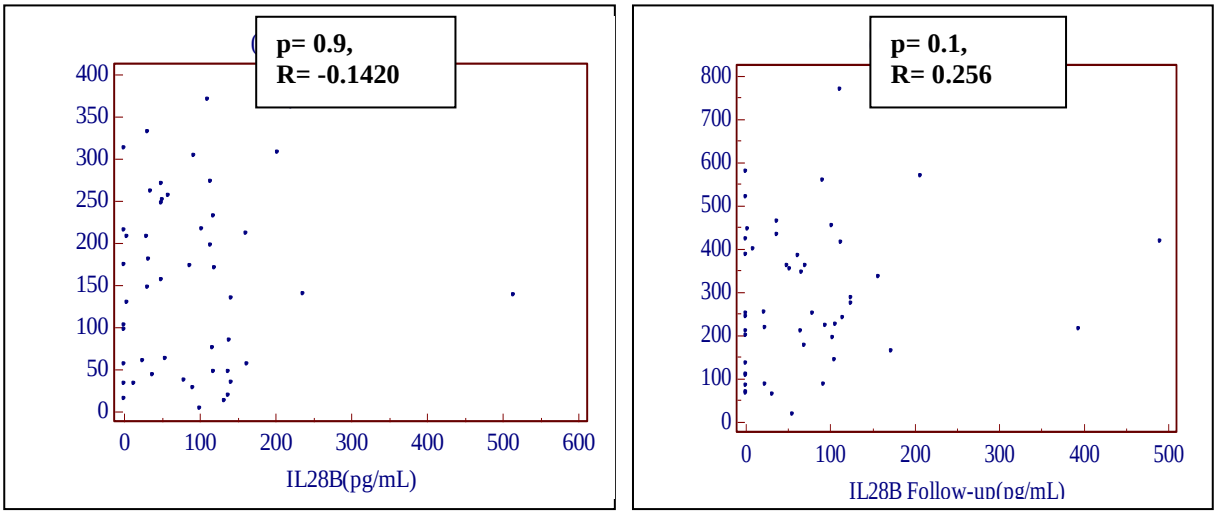


Figure 38: Scatter diagram showing the Spearman's correlation of IL28B plasma level with CD4+ T-cell counts before treatment and after 6-9 months of ART.

4.8.2. Association of IL-28B plasma level with CD4/CD3% counts before and after 6- 10 months of ART.

The association of IL-28B plasma level and absolute CD4/CD3% counts were analyzed using Spearman's correlation test. It was found that absolute CD4/CD3% is not significantly (p = >0.05 & R= -0.088) associated with median IL-28B plasma level before and after ART (p= >0.05 & R= - 0.11). IL-28B plasma level does not have any effect on CD4/CD3% before ART and increase in CD4/CD3% after 6-9 months of ART. The interpretation is summarized in the figure 39.

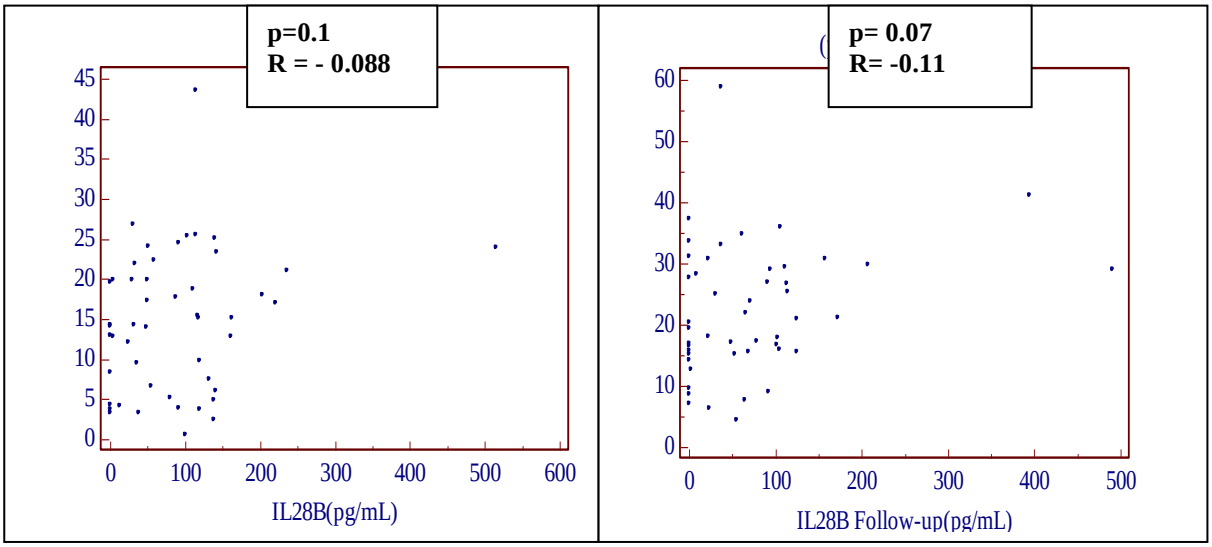


Figure 39: Scatter diagram showing the Spearman's correlation of IL28B plasma level with CD4/CD3% counts before treatment and after 6-9 months of ART.

4.8.3. Association of IL-28B plasma level with absolute CD8+ T-cell counts before and after 6-9 months of ART.

The association of IL-28B plasma level and absolute CD8+ T-cell counts were analyzed using Spearman's correlation test. It was found that absolute CD8+ T-cell count is not significantly (p = >0.05 & R= -0.057) associated with median IL-28B plasma level before and after ART(p=>0.05 & R = - 0.244). IL-28B plasma level does not have any effect on absolute CD8+ T-cell count before ART and increase in absolute CD8+ T-cell count after 6-9 months of ART. The interpretation is summarized in the figure 40.

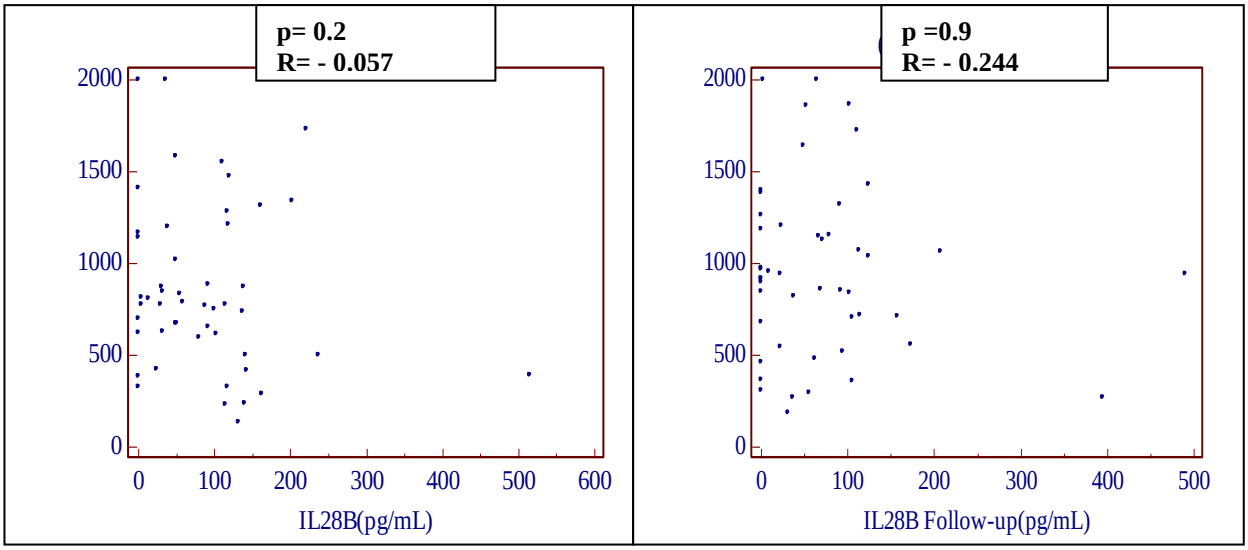


Figure 40: Scatter diagram showing the Spearman's correlation of IL28B plasma level with CD8+ T-cell counts before treatment and after 6-9 months of ART.

4.8.4.Association of IL-28B plasma level with CD8/CD3% counts

The association of IL-28B plasma level and absolute CD4/CD3% counts were analyzed using Spearman's correlation test. It was found that absolute CD4/CD3% is not significantly (p = >0.05 & R= -0.087) associated with median IL-28B plasma level before and after ART(p= >0.05 & R= -0.249). IL-28B plasma level does not have any effect on CD4/CD3% before ART and increase in CD4/CD3% after 6-9 months of ART. The interpretation is summarized in the figure 41.

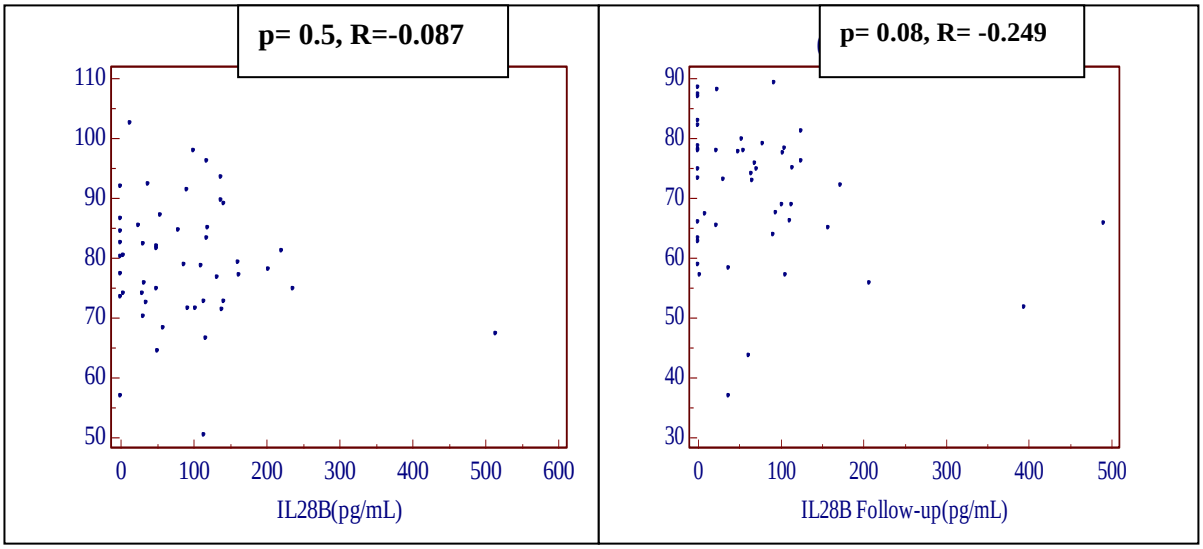


Figure 41: Scatter diagram showing the Spearman's correlation of IL28B plasma level with CD8/CD3% counts before treatment and after 6-9 months of ART.

4.8.5.Association of IL-28B plasma level with CD4/CD8 ratio before and after 6-9 months of ART in HIV infected individuals.

The association of IL-28B plasma level and absolute CD4/CD3% counts were analyzed using Spearman's correlation test. It was found that absolute CD4/CD3% is not significantly (p = >0.05 & R= -0.001) associated with median IL-28B plasma level before and after ART (p=>0.05 & R= - 0.042). The interpretation is summarized as scattered plot in the figure 42.

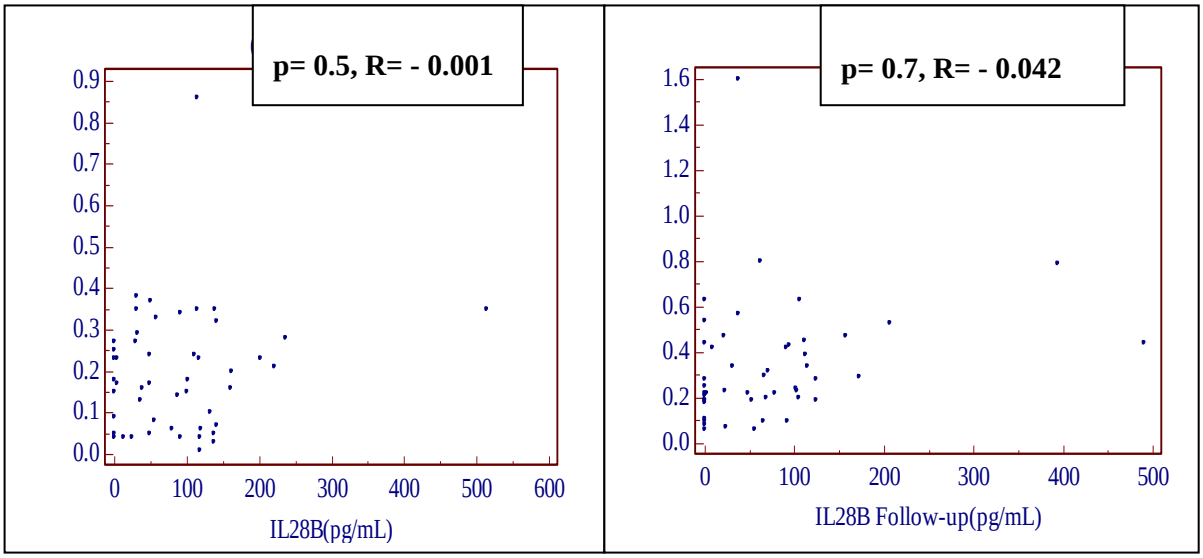


Figure 42: Scatter diagram showing the Spearman's correlation of IL28B plasma level with CD4/CD8 ratio before treatment and after 6-9 months of ART.

5. Discussion

Infection with HIV continues to be one of the major causes of mortality and morbidity especially in developing countries where the prevalence is high. The estimated number of people living with HIV worldwide is 35.3 million. As per the 2012 WHO report there are about 2.3 million newly infected individuals at the same time AIDS related deaths account for 1.6 million (2). The infection spreads through sexual, parenteral and from an infected mother to child. The progression of HIV disease is marked by progressive loss of CD4+ T cells, leading to AIDS and finally death. There are several innate and host factors that can affect the progression of the infection to AIDS(5). Anti Retroviral Therapy (ART) is the only treatment available to inhibit the viral replication, hence reducing the AIDS related mortality and morbidity through immune reconstitution. The response to ART is promising, when ART was introduced in India in 2004, the prevalence of HIV fell from 0.39% to 0.27% according to NACO annual report 2012-13, thus proving its major role in reducing the disease burden.

The CD4 + T cell and the HIV-1 viral load are the two independent predictors of the disease progression in an infected individual(179). Several studies have documented that compared to the zero percentage of five year risk of progression in an infected individual with CD4+ T cell counts of > 500 cells/ul the 5 year risk in an individual with a CD4+ T cell count of < 200 cells/ul is 81%(180). It is also documented that CD4 + T cell percentage is a better marker than the absolute count in individuals in whom CD4+ T cell count is > 350 cells/ul while in those with < 350 cells / ul. Another important factor that can affect the progression is the immune activation leading to higher number of activated T cells that can easily get infected by HIV(180).

There are several genetic factors that can also affect the rates of disease progression in treatment naïve HIV infected individuals. Among them the most important one identified is

HLA genotype. There are reports which associated rapid progression of the disease to AIDS among individuals with HLA alleles A24, B35, B37, B56, B58S and A1-B8-DR3 while long-term no progression is associated with HLA alleles B57, B27, B14 and C8(129).Other two important genetic factors well studied are CCR5-Δ32 and polymorphism in the gene encoding SDF1. In a multivariate logistic regression model it is reported that HIV infected individuals based on the presence or absence of heterozygous CCR5-Δ32, homozygous SDF1 and positive for HLA-B27 without HLA-DR6 but with at least three other HLA alleles (-A3, -B14,-B17, or -DR7) can be correctly classified as long-term nonprogressors (70 percent) or progressors (81 percent)(181). Yet another meta analysis showed that individuals with CCR5-Δ32 and CCR2-64I alleles had a lower level of viral load following seroconversion and there is a reduction in the risk towards to AIDS or death(182)

Recently there is lot of interest shown with the role of IL28B haplotype following the publication of 4 land mark study in 2009 showing that this can strongly affects the immune response in hepatitis C virus-infected patients.

Ge et al,(2009), 1,137 patients of the previously conducted IDEAL study were recruited , the genotype detection was done by Illumina Human610-quad BeadChip . They found that, in patients of European ancestery, African-Americans and Hispanic population the CC genotype is associated with a greater rate of SVR than the TT genotype(9).

Suppaiah et al, (2009) carried out a genome-wide association study (GWAS) of sustained virological response (SVR) to PEG-IFN-α/RBV combination therapy in 293 Australian individuals infected with genotype 1 hepatitis C virus (HCV). Among these 162 were non - responders and the remaining 131 were responders. They concluded that the rs8099917 G allele predicts non-response with sensitivity of 57% and specificity of 63%. The homozygous GG allele was associated with non response with a positive predictive value (failure to respond) of 64%(8).

In yet another genome-wide association study among 154 Japanese patients who were infected with HCV genotype 1 and non responders to PEG-IFN-α/RBV combination therapy showed that the G allele and T allele at the rs8099917 SNP was significantly associated with nil virological response(144).

Thomas *et al*(2009), in a GWAS assessed the association of rs12979860 SNP in 1008 individuals from 6 independent HCV cohorts. Among these individuals 388 were cleared the virus while remaining 620 were with persistent infection. The authors concluded that the protective effect of C is primarily recessive, as no significant difference was observed between the C/T and T/T genotypes among African Americans, Caucasians, or combined ethnic groups for clearance of HCV. The C/C genotype was consistently protective relative to C/T and/or T/T. HCV clearance was observed much more frequently than expected (53%) in the C/C group(183).

With these background information from all these HCV related studies our overall aim of the study was to explore the same kind of immune response in HIV infected individuals as well as HIV also is an RNA virus like HCV. Towards this we recruited 49 treatment naïve HIV-1 infected individuals who were eligible for ART initiation as there CD4+ T cell count was < 350 cells/ul. These individuals were followed up with one estimation of CD4+/CD8+/CD3+ T cell count following treatment in the next 6-10 months. Subsequently these cell counts, prior to and after treatment, IL-28 polymorphism and Il-28 plasma level were analyzed to see any association between immune reconstitution IL-28Bgenotype/haplotype at two positions; rs12979860 and rs8099917rs, In order to avoid too much bias in genetic background we have selected only subjects belong to the south Indian states as there were evidence that individuals from these states are genetically close(184). In order to look at whether these polymorphism has got any association with the susceptibility to HIV infection we also

recruited 30 age and sex matched HIV negative healthy controls who belongs to the south Indian states.

The IL-28B polymorphism was detected using PCR-RFLP method for genotyping of IL28B rs12979860 and rs8099917 SNPs is a cost effective method(185).The genotypes were obtained as CC,CT and TT of rs12979860 and TT,GT and GG of rs8099917 SNPs based on the digested products after treatment with restriction enzymes. In this study, among the HIV infected individuals the genotype frequency of the SNP rs12979860 CC genotype (wild) was higher 32 (65.3%) compared with 15(30.6%) of CT and 2(4.1%) TT genotypes. The frequency of SNP rs8099917 TT genotype (wild) was higher 39(79.6%) compared with 9(18.4%) of GT and 1(2%) of GG genotypes.

Among the 30 healthy controls in our study the genotype frequency of SNP rs12979860 CC genotype was higher 18(60%) compared with 10(33.3%) of CT and 2(6.7%) of TT genotypes. The frequency of SNP rs8099917 TT genotype was 23(76.7%) compared with 7(23.3%) of GT and GG genotype was not reported. The frequency distribution observed for SNP rs8099917 in our study for genotypes TT, GT were 76.7% 23.3% respectively. There were no individuals with G/G genotype observed in our HIV seronegative controls. The above frequency in the distribution of IL-28B genotypes in south Indian population is similar to another study which was conducted in Andhra Pradesh(186). The frequency reported out of 220 subjects recruited in that study was CC(59.09%), CT(34.09) and TT(6.81) for SNP rs12979860(186). In the study reported in this thesis there was no significant differences found in the distribution of IL-28B polymorphism at both the SNPs between HIV infected individuals and healthy controls ($p = < 0.5$). Frequency of haplotype in HIV infected individuals showed following distribution ; CC/TT 32(64.6%), CT/GT 8(16.7%), CT/TT 7(14.6%), TT/GG 1(2%) and TT/GT 1(2%).

The frequency of haplotypes in HIV seronegative controls were CC/TT 15(50%), CC/GT 3 (10%), CT/GT 3(10%), CT/TT 7(23.3%), TT/GT 1(3.3%) and TT/TT 1(3.3%). The frequency observed is similar to studies within India and outside.

Firdaus *et al* (2014), a study conducted in Kolkata, India reported that among the 400 HCV infected patients CC, CT and TT genotype at rs12979860 was seen in 70.75%, 24% and 5.25% respectively. The TT, GT and GG genotypes at rs8099917 was 77.5%, 15% and 7.50% respectively among the HCV infected patients. Among the 100 healthy controls in this study CC, CT and TT at rs12979860 was seen in 73%, 23% and 4% respectively and TT, GT and GG at rs8099917 was seen in 70%, 30% and 0% respectively(10).

Gupta *et al* (2014), a study conducted in New Delhi, India showed that among the HCV infected patients the distribution of CC, CT and TT at rs12979860 was 58.3%, 34.5% and 7.2%. The distribution among healthy controls was CC, CT and TT at rs12979860 was 60.6%, 35.2% and 4.2% respectively(158).

In an Iranian population the reported frequency of the rs12979860 CC, CT and TT genotypes were 40.4%, 47.1% and 12.5% and the frequency of the rs8099917 TT, GT and GG genotypes were 59.6%, 35.6% and 4.8%, respectively(190).

Studies reporting the effect of IL-28B polymorphism in HIV infected individuals are limited. Previous work by Sajadi et al,(2008) showed that IL-28B SNP was not associated with spontaneous HIV control in African American individuals(12). The frequency of the CC genotype was 12.5% in the Natural Virological Suppressors, 14.7% in the Low Viral Load cohort with 400–20,000 HIV-1 RNA copies/ml, 17.8% in the Medium High Viral Load cohort with >20,000 HIV-1 RNA copies/ml and 11.6% in an HIV-negative cohort. There was no statistical significance (p=.48) in the CC genotype distribution between these cohorts. There was no association observed between CC genotype distribution and HIV RNA viral load.

Salgado et al,(2011) showed that CC genotype of rs12979860 SNP does not contribute to HIV-1 control in a cohort of African–American elite controllers/suppressors(187). A cohort of 25 African-American elite controllers/suppressors, of whom 13 were seropositive for HCV were studied. Two of five patients who cleared HCV infection were positive for the C/C *IL28B* genotype. Overall, only 4 of 25 elite controllers/suppressors were positive for C/C genotype. This frequency was similar to that seen in HIV-1-infected patients with viral loads more than 10 000 copies/ml and seronegative patients at high risk for HIV infection in the ALIVE cohort. Similar frequencies were reported in other cohorts of HCV-infected African–Americans. There was lack of over representation of the protective C/C genotype in elite controllers/suppressors compared with HIV-1-infected patients with substantial levels of HIV-1 viremia which suggested that the *IL28B* SNP does not play a significant role in the elite control of HIV-1 replication.

Martin et al (2010) showed that the CC genotype of rs12979860 SNP does not affect the rate of CD4 +T-cell decline in HIV-1-infected patients. It not known whether it plays a role in the elite control of HIV-1 replication(151). The study included 226 individuals with HBV persistence, 384 with HBV recovery, and 2548 with or at high risk for HIV infection. They found out that the C/C genotype of rs12979860 was not associated with HBV recovery (odds ratio, 0.99), resistance to HIV infection (odds ratio, 0.97), or HIV disease progression (P >0.05). Hence they concluded that the IL28B single-nucleotide polymorphism affects the immune response to HCV but not to HBV or HIV.

The first study that showed a significant association between CC genotype and spontaneous control of HIV was by MachMach et al, (2013).There was no significant difference in viral load set point among the CC genotype and the CT, TT genotypes(188). In our study viral load estimation was not done as the primary objective of our study was to look at the effect of these polymorphisms on immune recovery and also the high cost involved with RNA

estimation. It is a well known fact that in HIV infected individuals at the end of 24 weeks (6 months) following ART there will not be any detectable viral RNA (5). However, estimation of the viral load prior to treatment could have given any association of IL-28 polymorphism and HIV-1 viral load.

In our study, the absolute CD4 + and CD8+ T-cells, CD4/CD8 were estimated both during recruitment and after 6-9 months of ART. There was a significant increase (p=< 0.0001) in the absolute CD4+ & CD8+T cell count, CD4/CD3% and reversal of CD4/CD8 ratio following 6-9 months of ART among HIV infected individuals as expected. This was well described in the literature(180).

There was no significant (p=>0.05) association between the rs12979860 SNP and the absolute CD4+ and CD8+ T-cell, CD4/CD3% and CD8/CD3% and CD4/CD8 ratio before initiation of ART. There was no significant (p= >0.05) association between the rs12979860 SNP and the increase in absolute CD4+ and CD8+ T-cell, increase in CD4/CD3% and decrease in CD8/CD3% and reversal of CD4/CD8 ratio after 6-9 months of ART.

However, the genotypic analysis showed that there is a significant(*p = 0.03*) association of CC genotype at rs12979860 when compared to other genotypes(C/T and T/T) with higher CD4+ T-cell count among treatment naïve HIV infected individuals and was also significantly (p= 0.05) associated with higher CD4+ T-cell count following 6-9 months of ART.

There was a no significant(p = >0.05) association of CC genotype at rs12979860 when compared to other minor genotypes(C/T and T/T) with CD8+ T-cell count among treatment naïve HIV infected individuals and was also not significantly (p= >0.05) associated with increase in CD8+ T-cell count following 6-9 months of ART.

There is a no significant(p = >0.05) association of CC genotype at rs12979860 when compared to other minor genotypes(C/T and T/T) with CD8/CD3% count among treatment

naïve HIV infected individuals and was also not significantly (p= >0.05) associated with decrease in CD8/CD8% count following 6-9 months of ART.

There is a no significant(p = >0.05) association of CC genotype at rs12979860 when compared to other minor genotypes(C/T and T/T) with CD4/CD8 ratio among treatment naïve HIV infected individuals and was also not significantly (p= >0.05) associated with reversal of CD4/CD8 ratio following 6-9 months of ART. The genotypic analysis of rs12979860 showed there is no significant(p = >0.05) association between the absolute CD4+ and CD8+ T-cell, CD4/CD3% and CD8/CD3% and CD4/CD8 ratio before initiation of ART. There was no significant (p= >0.05) association between the rs8099917 SNP and the increase in absolute CD4+ and CD8+ T-cell, increase in CD4/CD3% and decrease in CD8/CD3% and reversal of CD4/CD8 ratio after 6-9 months of ART. One of the biases that could have had at this finding may be because of the predominance of the CC genotype in the population. However if that is the case this significance could have for both SNPs at rs8099917 and rs129779860 SNP. Since it is present only with SNP this may be a true association.

In our haplotypic analysis we found that there was a significant (p=0.03) association of CC/TT haplotype of rs 12979860/rs8099917 (%) when compared to other haplotypes [(CT/GT) % and CT/TT) with higher CD4+ T-cell count among treatment naïve HIV infected individuals and was also significantly (p= 0.05) associated with higher CD4+ T-cell count following 6-9 months of ART. There was no significant (p = 0.1) association of CC/TT haplotype when compared to other haplotype with higher CD4/CD3% among treatment naïve HIV infected individuals, However they were associated with significant (p=0.03) higher CD4/CD3% compared to other haplotypes following 6-9 months of ART.

There was a no significant(p = >0.05) association of haplotypes with CD8+ T-cell count and CD8/CD3% among treatment naïve HIV infected individuals and was also not significantly (p= >0.05) associated with increase in CD8+ T-cell count and decrease in CD8/CD3% following 6-9 months of ART. There is a no significant (p = >0.05) association of haplotypes with CD4/CD8 ratio among treatment naïve HIV infected individuals and was also not significantly (p= >0.05) associated with reversal of CD4/CD8 ratio following 6-9 months of ART.

Out of 49 HIV infected individuals, one individual with a TT/GG haplotype presented with Immune reconstitution inflammatory syndrome (IRIS) with *Pneumocystis carinii* pneumonia. IRIS usually occur when the ART is initiated when the individual's CD4+ T cell count is very low and in this individual it was less than < 50 cells/ul. Whether individuals with homozygous haplotype at both the position are prone to get IRIS needs further investigation.

The influence of the IL-28B SNP on IL-28B (IFN-λ3) production and activity are still unknown. Suppaiah et al,(2009) reported that the T/T haplotype of rs8099917encompasses a region likely to affect expression of *IL28A* and *IL28B*. They found that in whole blood, the responder haplotype was weakly associated with higher expression levels(8). Most studies have concluded that the SNPs rs12979860 and rs8099917 are associated with reduced IFNλ3 expression during chronic HCV infection in liver biopsies, serum, and PBMCs(146)(144). There are no studies that has shown a significant increase in IFNλ3 expression due to a SNP to best of our knowledge. The SNPs may also affect the function of other genes in the locus. These variants have been associated with alteration in serum IFNλ1 and IFNλ2 levels and with HCV infection outcomes, both spontaneous resolution and IFN treatment responses(121).

In our study, there was no significant (>0.05) correlation observed between IL-28B genotypes and IL-28B plasma level among treatment naïve HIV infected individuals and

following 6-9 months of ART. There was no significant (p=>0.05) correlation between IL-28B haplotypes and the IL-28B plasma level before and after 6-9 months of ART.

However, out of 49 HIV infected individuals, 8 HIV infected indviduals with CT/GT haplotype had a higher median IL-28B plasma levels when compared to other haplotypes CC/TT, CT/TT, before initiation of ART. The median absolute CD4+ T-cell count values of these HIV infected were higher as observed with the CC/TT haplotype which had a significant(p=0.03) association with higher CD4+ T-cell counts before initiation of ART. There was a significant decrease in IL-28B plasma level following 6-9 months of ART.

RR Tian *et al*(2012), reported previously that HIV-1 infected individuals with CD4+ T-cell numbers greater than 800 cells/µL retained a similarly level of plasma IFN -λ1 to the uninfected controls and IL-λ1 was significantly elevated along with the depletion of CD4+ T-cells in HIV-1 infected individuals(119). The IFN -λ1 production was dropped when CD4 numbers were lower than 200/µL (known as the defined AIDS phase) the reason may be due to defective cytokine secretion or the persistent depletion of blood myeloid cells, which are the main producers of IFN-λ. The same investigators has also shown in vitro that that IFN-lamda has got an anti viral effect against HIV replication. However in vivo monitoring showed that plasma IL-29 level was increased along with the depletion of CD4+ T-cells in HIV-1 infected patients and the elevated IL-29 showed minimal inhibition of viral replication(119). This was also reported with experimental HBV infection among animals (109). The same phenomena maybe happening with HIV-1 infected individuals with CT/GT haplotype in our study.

High levels of IFNλs, but not IFN-α, is also observed during viral infection of lung and liver tissues, IFNλs seem to be the major IFNs induced in airway epithelial cells during infection with respiratory viruses Influenza A and Respiratory Synctitial virus(189). In our study the concurrent respiratory infections in HIV positive individuals were not analyzed. This may be

one of the reason for higher IL-28B plasma levels in patients with CT/GT haplotype and the reduction in the IL-28B plasma level following ART may be that the ART would have resolved the opportunistic respiratory infections following immune reconstitution.

Studies reporting the IL-28B plasma level in healthy individuals are limited. Pica et al(2010) reported the difference PBMC from patients with a history of recurrent Herpes labialis produced markedly lower levels of IFN-λ and marginally lower levels of IFN-α and IFN-γ than those from the history-negative HSV-1-seropositive controls(190).

In our study, there was no significant ($p = >0.05$) difference observed in IL-28B plasma level in treatment naïve HIV infected individuals and healthy controls ($p= >0.05$). Hence, there is no significant correlation between IL-28B polymorphism and IL-28B plasma level among treatment naïve HIV infected individuals and following 6-9 months of ART.

Studies reporting the effect of IL-28B on CD4+ T-cell counts are limited. In our study we looked at the association of IL-28B level with the CD4+, CD8+ absolute T-cell counts and CD4/CD8 ratio. We could not find any significant (>0.05) correlation between IL-28B plasma level with absolute CD4+ T-cell counts, CD4/CD3%, absolute CD8+ T-cell , CD8/CD3% and CD4/CD8 ratio among treatment naïve HIV infected individuals and following 6-9 months of ART.

In conclusion our preliminary data from this pilot study showed significantly higher CD4+ T-cells among HIV infected individuals with wild haplotype (CC/TT) prior to ART and significantly high CD4+ T cells and CD4/CD3% following ART. However, this preliminary study showed no association of IL-28B polymorphism(s) with IL-28B plasma level and CD4+ T cell count or CD4/CD8 ratio. Since IFN-λ is a powerful immune modulator

functional studies are warranted to understand the IFN-λ mediated immunopathogeneis in HIV infection. Furthermore studies with larger sample size are required to look at the association of IL-28B polymorphisms with plasma level and IL-28B mRNA expression among HIV infected individuals.

6. Summary

In this study a total of 80 HIV infected individuals were recruited after taking informed consent. Only 49 of the 89 recruited HIV infected individuals were followed for 6-10 months after initiation of ART. Among 80 recruited initially 7 patients expired during the study and 24 patients either did not follow up or were not initiated on treatment.

1) Out of 49 HIV infected individuals 30 were from Tamil Nadu of which 16 were males and 14 were females with mean age of 42 years (± 10). Eighteen were from Andhra Pradesh of which 12 were males and 6 were females with mean age of 41 years (± 10) and 1 male from Kerala. The 30 age and sex matched HIV negative healthy controls recruited were all from Tamil Nadu with mean age 42years(±10).
2) Among the 49 HIV infected individuals the genotype frequency of the SNP rs12979860 C/C genotype was higher 32 (65.3%) compared with 15(30.6%) of C/T and 2(4.1%) T/T genotypes. The frequency of SNP rs8099917 T/T genotype was higher 39(79.6%) compared with 9(18.4%) of G/T and 1(2%) of G/G genotypes. Among the 30 healthy controls the genotype frequency of SNP rs12979860 C/C genotype was higher 18(60%) compared with 10(33.3%) of C/T and 2(6.7%) of T/T genotypes. The frequency of SNP rs8099917 T/T genotype was 23(76.7%) compared with 7(23.3%) of G/T and G/G genotype was not reported. No significant (p= >0.05) differences were found out in the distribution of IL-28B polymorphism at both the SNPs between HIV infected individuals and healthy controls.
3) The above frequency in the distribution of IL-28B genotypes in south Indian population is similar to in the one already reported from Andhra Pradesh. The frequency reported out of 220 subjects recruited in that study was CC (59.09%), CT(34.09) and TT(6.81) for SNP rs12979860.

4) The absolute CD4 + and CD8+ T-cells, CD4/CD8 were estimated both during recruitment and after 6-9 months of ART. There was a significant increase (p=< 0.0001) in the median absolute CD4+ T cell count, CD4/CD3% and there was a reversal of median CD4/CD8 ratio following 6-9 months of ART among the HIV infected studied population.

5) There was a significant(*p = 0.03*) association of CC genotype at rs12979860 when compared to other genotypes(C/T and T/T) with higher CD4+ T-cell count among treatment naïve HIV infected individuals and was also significantly (p= 0.05) associated with higher CD4+ T-cell count following 6-9 months of ART

6) There is a no significant(p = >0.05) association of CC genotype at rs12979860 when compared to other minor genotypes(C/T and T/T) with CD8+ T-cell count and CD8/CD3% among treatment naïve HIV infected individuals and was also not significantly (p= >0.05) associated with increase in CD8+ T-cell count following 6-9 months of ART

7) There was a no significant(p = >0.05) association of CC genotype at rs12979860 when compared to other genotypes(C/T and T/T) with median CD4/CD8 ratio among treatment naïve HIV infected individuals and was also not significantly (p= >0.05) associated with reversal of CD4/CD8 ratio following 6-9 months of ART.

8) In contrast to the association seen between the CD4+ T cell count and the wild genotype at rs12979860 there was no correlation observed between the genotypes at rs8099917 and CD4/CD8 + T-cell counts prior to or following ART.

9) There was a significant(p=0.03) association of CC/TT haplotype at when compared to other haplotypes (CT/GT and CT/TT) with higher CD4+ T-cell count among treatment naïve HIV infected individuals and was also significantly (p= 0.05) associated with higher CD4+ T-cell count following 6-9 months of ART.

10) There was no significant (p = 0.1) association of CC/TT haplotype when compared to other haplotype with higher CD4/CD3% among treatment naïve HIV infected individuals, However they were significantly(p = 0.03) associated with higher CD4/CD3% compared to other haplotypes following 6-9 months of ART.
11) There was a no significant(p = >0.05) association of haplotypes with CD8+ T-cell count and CD8/CD3% among treatment naïve HIV infected individuals and was also not significantly (p= >0.05) associated with change in CD8+ T-cell count and decrease in CD8/CD3% following 6-9 months of ART.
12) There was a no significant(p = >0.05) association of haplotypes with CD4/CD8 ratio among treatment naïve HIV infected individuals and was also not significantly (p= >0.05) associated with reversal of CD4/CD8 ratio following 6-9 months of ART.
13) There was no significant (p = >0.05) difference in the median IL-28B plasma level among treatment naïve HIV infected individuals and healthy controls
14) There was no significant (>0.05) correlation observed between IL-28B plasma level with absolute CD4+ T-cell counts and CD4/CD3% among treatment naïve HIV infected individuals and following 6-9 months of ART
15) There was no significant (>0.05) observed correlation between IL-28B genotypes at both rs12979860 & rs8099917 with IL-28B plasma level among treatment naïve HIV infected individuals and following 6-9 months of ART.
16) There was no significant (p=>0.05) correlation observed between IL-28B haplotypes between the IL-28B plasma level before and after 6-9 months of ART.
17) When we looked at the association of the haplotypes and the IL-28B plasma level, we found out that the CT/GT haplotype had a significant higher IL-28B plasma level compared to wild type CC/TT before the initiation of ART though the median CD4+ T-cell counts were almost similar. Following 6-10 months of ART the median

decrease observed in CT/GT haplotype compared to CC/TT wild type were significant.The reason for this needs to be further investigated.

18) In conclusion our preliminary data from this pilot study showed significantly higher CD4+ T-cells among HIV infected individuals with wild haplotype (CC/TT) prior to ART and significantly high CD4+ T cells and CD4/CD3% following ART. However, this preliminary study showed no association of IL-28B polymorphism (s) with IL-28B plasma level and CD4+ T cell count or CD4/CD8 ratio. Since IFN is a powerful immune modulator functional studies are warranted to understand the IFN lambda mediated immuno-pathogeneis in HIV infection. Furthermore studies with larger sample size are required to look at the association of IL-28B polymorphisms with plasma level and IL-28B mRNA expression among HIV infected individuals.

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Consent Form

INFORMED CONSENT

Information sheet

Title of study –

"A pilot study to look at the effect of IL-28 polymorphism on IL-28 expression and immunological recovery among HIV infected individuals following ART"

Description of the study

HIV is one of the important infections worldwide. Over 2.4 million people are infected with HIV in India which stands 3rd in the world. It is transmitted through unsafe sexual intercourse, infected blood and blood products and also from infected mother to the child. The disease progresses by destroying the immune system (CD4+ T lymphocytes) in the body and thus causes infections by several other microorganisms ultimately leading to death. Since there is no permanent cure for HIV infection, it is important to control the disease progression with drugs that stop viral multiplication. The Anti-Retroviral Therapy (ART) is the only available treatment to control progression. The response to these drugs is monitored through estimation CD4+ and HIV-1 viral load. Cytokines like Interleukin-28 are group of proteins which play an important role in the multiplication of virus and progression of disease. CD4 + T lymphocytes play an important role in the human body to fight against infections. Experiments have shown that a change in the gene that controls Interleukin-28 can improve the condition in patients with Hepatitis C infection which infect liver. In this study we are going to look at this change can improve the health condition of HIV infected individuals.

Note : If you are participating as a control (healthy individual) in the study, HIV testing will be done prior to recruitment, towards this a separate consent as per national guidelines(attached)will be obtained. We will be collecting 8ml of blood towards HIV testing , CD4+/CD8+ T-cell count,IL-28 plasma level and IL-28 polymorphism .

If you are found HIV positive, result will be informed and you will be referred to ID clinic for counselling and further management and you will not be part of study.

Can you withdraw from this study after it starts and during follow up?

Your participation in this study is entirely voluntary and you are also free to decide to withdraw permission to participate in this study.

What will happen if you develop any study related injury?

We do not expect any injury to happen to you if you participate in the study.

Will you have to pay for the study?

No

What amount of sample is collected?

Extra 5-6ml of blood is collected if you are coming for routine testing

What happens after the study is over?

The results obtained from the samples collected will be used only for the study and data may publish in scientific journal and the remaining samples will be stored in our laboratory. All the details will be kept confidential including the results of the test for HIV.

Will your personal details be kept confidential?

The results of this study will be published in a medical journal but your identity will not be revealed in any publication or presentation of results.

If you have any further questions, please ask Dr.Srinidhi.B.V. (Department of Microbiology)(Contact number:+919597557036,email id:newtonnidhi@gmail.com)

CONSENT FORM TO TAKE PART IN THE STUDY

Study title: "A pilot study to look at the effect of IL-28 polymorphism on IL-28 expression and immunological recovery among HIV infected individuals following ART"

Study Number: **Hosp. no**:

Participant's name:

Date of Birth / Age (in years):

I_____

_____, son/daughter of _____

(Please tick boxes)

Declare that I have read the information sheet provided to me regarding this study and have clarified any doubts that I had. [ ]

I also understand that my participation in this study is entirely voluntary and that I am free to withdraw permission to continue to participate at any time without affecting my usual treatment or my legal rights [ ]

I understand that the study staff and institutional ethics committee members will not need my permission to look at my health records even if I withdraw from the trial.

I agree to this access [ ]

I understand that my identity will not be revealed in any information released to third parties or published [ ]

I voluntarily agree to take part in this study [ ]

Would you like to give permission to store the remaining sample in our specimen bank and can be used for future studies [YES/NO]

Healthy individuals, I also understand that sample collected will be tested for HIV [Yes/No]

Name: Name of witness:

Signature/Thumb impression: Signature of witness:

Date: Relation to participant:

Signature of investigator:

Date:

Patient Data Sheet

STUDY NO	State	AGE	SEX	MARITAL S	CD4	CD4%	CD8%	CD8	CD3	CD4/CD8	IL28B	CD4F	CD4F%	CD8F	CD8F%	CD3F	CD4/CD8F	IL-28BF	rs12979860	rs8099917	ART
HIVILP1	TN	49	M	MARRIED	313	19.66	73.37	1168	1592	0.27	796.135	416	26.79	1069	68.83	1553	0.39	112.887	CC	TT	ZLN
HIVILP3	TN	37	F	MARRIED	261	9.46	72.49	2000	2759	0.13	1379.565	362	17.12	1642	77.67	2114	0.22	48.2715	CC	TT	ZLN
HIVILP4	TN	38	F	MARRIED	28	3.90	91.36	656	718	0.04	359.02	243	15.84	1260	82.14	1534	0.19	0	CT	TT	ZLE
HIVILP5	TN	45	M	UNMARRIE	362	17.02	81.24	1728	2127	0.21	1063.605	361	23.94	1128	74.80	1508	0.32	70.794	CC	TT	ZLN
HIVILP9	AP	36	M	MARRIED	304	24.54	71.59	887	1239	0.34	619.67	417	29.12	942	65.78	1432	0.44	490.2595	CC	TT	ZLN
HIVILP12	AP	55	M	MARRIED	197	43.49	50.33	228	453	0.86	226.93	135	27.72	308	63.24	487	0.44	111.162	CT	GT	TFV,FTC,EFV
HIVILP10	TN	28	F	MARRIED	216	12.92	84.33	1410	1672	0.15	836.075	769	29.47	1724	66.08	2609	0.45	0	CC	TT	ZLE
HIVILP14	AP	65	F	MARRIED	48	4.92	89.55	874	976	0.05	488.025	107	9.58	975	87.29	1117	0.11	0	TT	GG	TLN
HIVILP15	TN	56	F	MARRIED	273	25.59	72.63	775	1067	0.35	533.675	434	58.81	272	36.86	738	1.6	36.942	CC	TT	ZLN
HIVILP16	AP	41	F	MARRIED	57	15.20	77.07	289	375	0.2	187.6	163	21.11	557	72.15	772	0.29	172.507	CC	TT	ZLN
HIVILP17	AP	23	F	MARRIED	19	2.41	93.52	736	787	0.03	393.515	86	9.02	851	89.30	953	0.1	394.3005	CC	TT	ZLN
HIVILP20	AP	40	M	MARRIED	34	6.03	89.01	502	564	0.07	282.035	254	30.68	542	65.46	828	0.47	105.019	CC	TT	TDF/3TC/EFV
HIVILP22	TN	39	M	MARRIED	212	12.82	79.32	1312	1654	0.16	827.08	453	16.72	1866	68.88	2709	0.24	21.932	CT	GT	ZLE
HIVILP23	AP	49	M	MARRIED	13	7.56	76.74	132	172	0.1	86.05	17	4.44	298	77.81	383	0.06	101.532	CT	GT	ZLN
HIVILP25	AP	39	F	MARRIED	135	23.40	72.62	419	577	0.32	288.66	521	33.70	968	62.61	1546	0.54	55.317	CC	TT	ZLN
HIVILP26	AP	45	M	MARRIED	370	18.75	78.66	1552	1973	0.24	986.62	423	31.10	897	65.96	1360	0.06	0	CC	TT	ZLN
HIVILP28	TN	55	M	MARRIED	174	14.23	56.99	697	1223	0.25	611.625	384	34.72	482	43.58	1106	0.8	0	CC	TT	TLN
HIVILP29	TN	55	M	MARRIED	33	4.19	102.41	806	787	0.04	393.52	84	15.14	460	82.88	555		104.276	CC	TT	ZLN
HIVILP33	AP	41	M	MARRIED	76	15.48	66.60	327	491	0.23	245.615	225	35.94	358	57.19	626	0.63	0	TT	GT	ZLN
HIVILP34	TN	34	F	MARRIED	181	21.94	75.76	625	825	0.29	412.645	337	30.80	711	64.99	1094	0.47	8.566	CC	TT	ZLN
HIVILP36	TN	42	M	MARRIED	4	0.52	97.91	750	766	0.15	383.075	224	29.02	521	67.49	772	0.43	105.659	CC	TT	TLE
HIVILP37	TN	33	F	MARRIED	232	15.10	83.27	1279	1536	0.01	768.005	110	7.03	1385	88.50	1565	0.08	157.112	CC	TT	ZLN
HIVILP43	TN	51	M	MARRIED	217	25.32	71.53	613	857	0.18	428.59	560	27.01	1323	63.82	2073	0.42	94.279	CC	TT	ZLN
HIVILP44	AP	36	M	UNMARRIE	147	14.33	82.26	844	1026	0.35	513.175	242	25.37	716	75.05	954	0.34	0	CT	GT	ZLN
HIVILP47	AP	34	F	MARRIED	130	12.90	80.46	811	1008	0.17	504.085	199	17.01	914	78.12	1170	0.22	91.277	CC	TT	ZLN
HIVILP48	TN	26	F	MARRIED	43	3.32	92.28	1196	1296	0.16	648.08	194	17.96	838	77.59	1080	0.23	114.5555	CC	TT	ZLE
HIVILP49	TN	35	F	MARRIED	60	12.05	85.34	425	498	0.04	249.02	209	7.73	2000	73.99	2703	0.1	0	CC	TT	TLE
HIVILP50	TN	55	M	MARRIED	173	17.78	78.83	767	973	0.14	486.57	287	20.99	1040	76.08	1367	0.28	102.366	CC	TT	ZLE
HIVILP51	TN	60	M	MARRIED	208	19.85	73.95	775	1048	0.23	524.115	346	22.00	1145	72.79	1573	0.3	64.825	CT	GT	
HIVILP52	AP	60	M	MARRIED	208	19.85	73.95	775	1048	0.27	524.135	219	18.08	943	77.87	1211	0.23	124.657	CC	TT	
HIVILP53	TN	48	M	MARRIED	98	3.79	77.25	2000	2589	0.05	1294.525	353	15.15	1860	79.83	2330	0.19	65.983	CC	TT	
HIVILP54	TN	23	F	MARRIED	271	13.98	81.63	1582	1938	0.05	969.025	386	20.29	1395	73.34	1902	0.28	22.2015	CT	TT	
HIVILP55	TN	46	M	MARRIED	156	17.33	74.89	674	900	0.17	450.085	275	15.59	1431	81.12	1764	0.19	52.4835	CT	TT	
HIVILP56	TN	47	F	MARRIED	33	4.38	82.49	622	754	0.23	377.115	447	12.77	2000	57.14	3500	0.22	0	CT	GT	
HIVILP59	TN	37	M	MARRIED	251	24.09	64.49	672	1042	0.37	521.185	465	33.03	820	58.24	1408	0.57	124.565	CC	TT	
HIVILP63	KER	51	M	MARRIED	257	22.35	68.35	786	1150	0.33	575.165	580	37.30	914	58.78	1555	0.63	2.5195	CC	TT	
HIVILP64	TN	25	F	MARRIED	15	3.37	86.52	385	445	0.04	222.52	69	14.23	363	74.85	485	0.19	37.275	CT	TT	
HIVILP66	AP	53	M	MARRIED	140	21.05	74.74	497	665	0.28	332.64	64	25.00	187	73.05	256	0.34	0	CC	TT	
HIVILP67	TN	34	F	MARRIED	308	17.97	78.00	1337	1714	0.23	857.115	569	29.81	1064	55.74	1909	0.53	0	CC	TT	
HIVILP70	AP	36	M	MARRIED	247	19.84	81.85	1019	1245	0.24	622.62	252	17.25	1154	78.99	1461	0.22	30.85	CT	GT	
HIVILP71	TN	39	M	MARRIED	37	5.25	84.54	596	705	0.06	352.53	176	15.49	861	75.79	1136	0.2	207.138	CT	GT	
HIVILP73	TN	35	M	UNMARRIE	170	9.80	84.96	1474	1735	0.06	867.53	210	19.34	845	77.81	1086	0.25	78.578	CC	TT	
HIVILP74	TN	32	M	MARRIED	63	6.60	87.21	832	954	0.08	477.04	67	8.57	679	86.83	782	0.1	68.8625	CC	TT	
HIVILP76	AP	45	M	MARRIED	103	8.31	91.85	1138	1239	0.09	619.545	251	16.63	1187	78.66	1509	0.21	0	CC	TT	
HIVILP80	TN	59	F	MARRIED	47	3.73	96.11	1210	1259	0.04	629.52	86	6.27	1207	88.04	1371	0.07	0	CT	TT	
HIVILP82	TN	40	M	MARRIED	57	14.11	80.20	324	404	0.18	202.09	560	23.29	1768	73.54	2404	0.32	0	CC	TT	
HIVILP83	AP	41	M	MARRIED	138	23.88	67.30	389	578	0.35	289.175	216	41.06	272	51.71	526	0.79	23.055	CT	TT	

Control Data Sheet

Controls	rs1297986(	rs8099917	IL-28B Plasma
HIVILC1	CC	TT	80.441
HIVILC2	CC	TT	143.047
HIVILC3	CC	TT	115.098
HIVILC4	CC	TT	101.6825
HIVILC5	CT	TT	68.143
HIVILC6	CT	GT	40.1935
HIVILC7	CT	TT	43.5475
HIVILC8	TT	GT	56.9635
HIVILC9	CC	TT	139.6935
HIVILC10	CC	TT	1010.594
HIVILC11	CC	TT	0
HIVILC12	CC	TT	16.154
HIVILC14	CT	GT	67.195
HIVILC15	CT	GT	68.6775
HIVILC16	CC	TT	7.618
HIVILC17	CC	TT	33.921
HIVILC18	CT	TT	187.661
HIVILC19	CC	TT	3.809
HIVILC20	CC	TT	42.0835
HIVILC21	CC	GT	140.8115
HIVILC22	TT	TT	196.71
HIVILC23	CT	TT	77.087
HIVILC24	CC	GT	53.6095
HIVILC25	CT	TT	97.2105
HIVILC26	CC	GT	50.2555
HIVILC27	CC	TT	33.486
HIVILC28	CT	TT	43.548
HIVILC29	CT	TT	7.799
HIVILC30	CC	TT	1.0915